Regulation of genes associated with avian B cell receptors involved in innate and adaptive immunity

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ABSTRACT

Epigenetics is a group of mechanisms associated with gene expression control. One of these mechanisms is methylation of cytosines which are part of a cytosine-guanine (CpG) dinucleotide. When this happens in the promoter region of a gene, it will control unwanted expression. For this process to take place, a methyl group donor is needed. One of the principal nutritional compounds that fit this role is folic acid (FA).

Our aim in this research was to examine whether exposure to FA would change the methylation profile and expression of toll like receptor (TLR) 2, immunoglobulin (Ig) β and major histocompatibility class (MHC) II β chain in chicken B cells.

The initial experiment used the chicken B cell derived DT-40 cell line. Cell cultures were incubated with 0, 1.72 or 3.96 mM of FA for 4 or 8 hours, with the 8 hour group treated with 0, 1 or 10 μg/ml of Lipopolysaccharide (LPS). A positive association was found between FA concentration and percent of Igβ promoter methylation and a negative association between FA concentration and percent of MHCII β chain promoter methylation after 4 hours of incubation. The expression of those two genes was affected as well. Both were downregulated after 8 hours compared to the 4 hours group. Incubation with 1.72 mM FA for 4 hours upregulated TLR2b and 3.96 mM FA for 4 hours upregulated MHCII β chain expression. FA at 3.96 mM for 8 hours downregulated TLR2b and upregulated Igβ expression. Treatment with LPS downregulated TLR2b expression. We concluded that FA indeed has an immunomodulatory effect on chicken B cells in a way that may possibly affect their ability to both recognize antigen through the TLR and B cell receptor (BCR) pathways and their ability to present antigen in an MHCII context.

A second series of experiments examined the effect of FA on embryonic B cells extracted from the bursa of Fabricius (BoF) at days (ED) 15, 18 and 21 of embryonic development. A novel protocol was

developed to obtain optimal growth and survival of the cells. The amount of chicken serum in the growth media was doubled and time of *ex vivo* culture was determined to ensure that the cells survive for the duration of the experiment. We found that our modified growth media maintained cells for the 4 hour duration of the experiment. When compared to cells that were not incubated (0 hours), cells harvested at ED15 and incubated for 4 hours had a lower population of $IgM^{low/med}$ cells, increased population of IgM^{high} cells and upregulated Igβ expression. When the cells were harvested at ED21 and incubated for 4 hours TLR2b, MHCII β chain as well as the expression of the reduced folate carrier (RFC), a FA transporter were downregulated compared to 0 hours. Exposure to FA did not have an effect on cell viability. For cells harvested at ED15 both 1.72 and 3.96 mM FA for 4 hours caused a downregulation of Igβ and the 3.96 mM treatment reduced the population of IgM^{med} cells when compared to these values after 4 hour incubation with no FA (0 mM FA). At ED18 both FA treatments reduced the IgM^{med} population and the 1.72 mM FA treatment reduced RFC expression compared to the 0 mM FA group. At ED21, 3.96 mM FA increased the IgM^{high} population. The methylation status of the promoter region of all examined genes was not affected by either removal of the cells from the BoF or treatment with FA. In this experiment, we demonstrated the immune modulating capabilities of FA in the developing B cell, and provided a protocol for maintaining the viability of these cells *ex vivo* for longer periods then previously published.

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LIST OF ABBREVIATIONS AND ACRONYMS

Abbreviation	Term
5mC	5-methyl cytosine
5-mTHF	5-methyltetrahydrofolate
ANOVA	Analysis of variance
APC	Antigen presenting cell
ATCC	American Type Culture Collection
BAFF	B-cell activating factor
Bax	Bcl-2-associated X protein
BCR	B cell receptor
BoF	Bursa of Fabricius
BSA	Bovine serum albumin
C/EBPα	CCAAT/enhancer-binding protein alpha
CD	Cluster of differentiation
cDNA	Complementary deoxyribonucleic acid
Ch	Chicken
ChB6	Chicken B cell marker B6 also known as Bu-1
CIITA	Class II transactivator
CO_2	Carbon dioxide
Conc.	Concentration

CPE	Cytoplasmic polyadenylation element
CpG	Cytosine-Guanine dinucleotide
CRE-BP1	Recombination causing binding protein 1
CTL	C-type lectin
CXCL	CXC motif ligand
CXCR	CXC motif chemokine receptor
DC	Dendritic cells
DMEM	Dulbecco's Modified Eagle's Medium
DNA	Deoxyribonucleic acid
DNMT	Deoxyribonucleic acid methyltransferases
ED	Embryonic day
EDTA	Ethylenediaminetetraacetic acid
ET	E26 transformation-specific protein
FA	Folic acid
FAS	First apoptotic signal
FITC	Fluorescein isothiocyanate
GI	Gastrointestinal
HLA	Human leukocyte antigen
ICAM-1	Intercellular adhesion molecule 1
IFN-γ	Interferon gamma
Ig	Immunoglobulin
IgG	G class immunoglobulin
IgM	M class Immunoglobulin

Igα	B cell antigen receptor complex associated protein alpha chain
Igβ	B cell antigen receptor complex associated protein beta chain
IL	Interleukin
ITAM	Immunoreceptor tyrosine based activation motif
LBP	Lipopolysaccharide binding protein
LFA-1	Lymphocyte function-associated antigen 1
LTA	Lipotechoic acid
LPS	Lipopolysaccharide
MAMP	Microbe associated molecular pattern
MBD	Methylated DNA binding protein
MeCP	Methyl CpG binding protein
MD2	Lymphocyte antigen 96
MHC	Major histocompatibility complex
mRNA	messenger RNA
miRNA	Micro RNA
MYD88	Myeloid differentiation factor 88
NaCl	Sodium Chloride
NaOH	Sodium hydroxide
NCBI	National Center for Biotechnology Information
NF-κB	Nuclear factor kappa B
NOD	Nucleotide-binding oligomerization domain
NRC	National Research Council
Pax	Paired box protein

PBS	Phosphate buffered saline
PCD	Programmed cell death
PCFT	Proton coupled folate carrier
PCR	Polymerase chain reaction
PI	Propidium iodide
Pop.	Population
PRR	Pattern recognition receptor
RFC	Reduced folate carrier
RIG	Retinoic acid inducible gene
RNA	Ribonucleic acid
SAM	S-adenosylmethionine
SDS	Sodium dodecyl sulfate
SE	Standard error
SEM	Standard error of the means
sIg	Surface immunoglobulin
SP	Specific protein
TCR	T cell receptor
TE	TRIS-EDTA
TF	Transcription factor
Th.	Helper T cell-like response
THF	Tetrahydrofolate
TLR	Toll like receptor
TNFα	Tumor necrosis factor alpha

TRIS	Tris(hydroxymethyl)aminomethane
YT	Yeast-Tripton

Standard units of measurements:

Abbreviation	**Term**
°C	Degrees (Celsius)
µg	Microgram
µl	Microliter
µM	Micromolar
µm	Micrometer
bp	Base pairs
Ct	Cycle threshold
FL	Fluorescence levels
h	Hours
kg	Kilogram
mg	Milligram
Min	Minute
ml	Milliliter
mM	Millimolar
N	Normal
ng	Nanogram
nm	Nanometer
pH	Hydrogen ion activity

1. General Introduction

1.1 The avian primary lymphoid tissue

The chicken as an immune system model was instrumental in important findings in immunological research. The discovery of vaccines by attenuating *Pasteurella multocida* by Louis Pasteur (Plotkin, S.A. 2003), *in ovo* vaccinations (Sharma, J.M. and Burmester, B.R. 1982) and allograft rejection (Simonsen, M. 1957), are examples which improved countless human and animal lives. Throughout

As chickens and mammals evolved from the same reptilian ancestor (Seto, F. 1981), there are similarities in lymphocyte development between the mammalian and avian immune systems, such as the T cell development process (Chen, C.H. et al. 1994). Hematopoietic stem cells, originating in the chicken from the para-aortic foci and the bone marrow (Dunon, D. et al. 1999) colonize the thymus in three waves. The cells then go through receptor rearrangement (Pickel, J.M. et al. 1993), after which the T cells emigrate from the thymus and populate the periphery and the spleen (Dunon, D. et al. 1997). Conversely, the development and maturation process of chicken B cells is different from the one described in mammals. While in mammals this process takes place in the fetal liver and then in the bone marrow (Melchers, F. 2015), the chicken has a different specialized organ for that purpose named the bursa of Fabricius (BoF) (Ciriaco, E. et al. 2003).

1.1.1 BoF development

The BoF is located between the cloaca and the sacrum and is attached to the gastrointestinal (GI) tract by the bursal duct (Casteleyn, C. et al. 2010). It was found to be an exclusive site for B cell maturation (these cells were in fact named B cells after this organ) in the chicken (Cooper, M.D. et al. 1965), and was the basis for the discovery of the role of the bone marrow in mammals in B cell maturation (Abdou, N.I. and Abdou, N.L. 1972).

During embryonic development the BoF is formed by interaction with the epithelium and mesenchyme (much like the thymus) (Olah, I. et al. 1986). The sac like formation is visible as early as embryonic development day (ED) 5 (Olah, I. et al. 1986). Over the next two days a lumen is formed, which is then connected to the proctodeum by the bursal duct (Nagy, N. and Olah, I. 2010). Further proliferation of epithelium and mesenchymal cells cause the formation of folds, usually around 12 of them, each with its own artery and vein. It is during this time, between ED10 and ED15, when the BoF is colonized by precursors of B cells (Nagy, N. et al. 2004). Each follicle is initially colonized by 2-3 B cell precursors, which continue to proliferate (Pink, J.R. et al. 1985).

By hatch, 90% of cells found in the BoF are immunoglobulin (Ig)M positive (McCormack, W.T. et al. 1991). B cells migrate from the follicles to an area where the bursal cortex is developed (Sayegh, C.E. et al. 2000), and from there they migrate to the periphery and secondary lymphoid tissues (Paramithiotis, E. and Ratcliffe, M.J. 1994a). The BoF, like the thymus, (Ciriaco, E. et al. 2003) goes through a process of involution, beginning at 3 months post hatch up until 7 months post hatch (Naukkarinen, A. and Sorvari, T.E. 1984). This site, whose sole purpose is B cell maturation and development, makes the chicken a prime candidate for the study of these cells.

1.2 B cell

Classically, B cells fill a crucial role in the adaptive immune system. B cells recognize antigens through their B cell receptor (BCR) (Paus, D. et al. 2006). This type of recognition leads to presentation of the processed antigen on major histocompatibility complex (MHC) proteins (Avalos, A.M. and Ploegh, H.L. 2014). Surrounding the germinal centers are T cell zones, where antigen presenting cells activate cluster of differentiation (CD) 4 positive naïve T cells (Zynda, E.R. et al. 2015). T cell receptors (TCR) are designed to recognize antigen presentation only in MHCII proteins (Banchereau, J. and Steinman, R.M. 1998). If they are activated and come into contact with B cells presenting the same antigen through the MHCII complex. B cell will go through several changes which would enhance the antibody mediated response against that antigen (Lanzavecchia, A. 1985). This interaction does not involve the MHCII and TCR alone, but also the CD4 co-receptor, which is needed for intercellular signaling (Abraham, R.T. and Weiss, A. 2004).

Another pair of receptors integral for the activity of the B cell are the CD40 ligand on the T cell, and its corresponding CD40 receptor on the B cell (Senhaji, N. et al. 2015), and finally lymphocyte function-associated antigen 1 (LFA-1) on the T cell which binds to intercellular adhesion molecule 1 (ICAM-1) on the B cell, and helps anchor these two cell types together (Shekhar, S. and Yang, X. 2012). The interaction between T and B cells, as well as the cytokine profile secreted by the T cell, induces somatic hypermutation which utilizes point mutations to increase the affinity of the B cell receptor to the antigen (Li, Z. et al. 2004). The interaction between the cells also induces class switching which changes the type of the antibody heavy chain produced by the B cells,

dictated by the nature of the insult (Hu, J. et al. 2013). The above mentioned interaction also induces proliferation of the B cells and differentiation into plasma cells, which secrete the high affinity, class switched antibody (Hu, Y. et al. 2013), and memory B cells, which do not secrete antibodies. However, if there is a second interaction with the same antigen, memory B cells will produce affinity matured, class switched antibodies (Ettinger, R. et al. 2005).

Through the interactions described above, the B cell develops all the traits of the adaptive immune system including highly specific (Weng, N.P. 2006), memory inducing response. This response will be faster and more potent following a second insult (Weng, N.P. 2006). For this type of response antigen is recognized by the BCR and presented in an MHCII context. Recognizing a specific antigen through the BCR, processing it and presenting it to an effector T cell in an MHCII context (a T dependent B cell activation) is only one way in which the B cell can be activated (Hu, J. et al. 2013). Alternatively, B cells can also respond in a relatively fast, low specificity manner with no change from first to second antigen interaction (Kelly-Scumpia, K.M. et al. 2011). Those are all traits of the innate immune response, which does not require interaction with a T cell – T independent B cell activation (Liu, Y.J. et al. 1991). T independent antigens, such as lipopolysaccharides (LPS), do not have to be processed and presented to T cells to elicit antibody production (Mamchak, A.A. and Hodgkin, P.D. 2000). These microbial associated molecular patterns (MAMP) can be recognized by toll like receptors (TLRs) on B cells without involving the BCR or the MHC II system. This recognition will result in the production of low affinity antibodies (Pone, E.J. et al. 2015). Furthermore, recognition of MAMPs by TLRs can be followed by a cross linking and engagement between the TLRs and BCR, which can lead to antibody class switching and DNA recombination in both T cell independent (Macpherson, A.J. et al. 2000) and dependent

(Matter, M.S. and Ochsenbein, A.F. 2008) manners. Herein lays the elegance and importance of the B cell in defending the organism. The different receptors expressed by the B cell can work, alone or together, to allow a full response spectrum in terms of specificity, speed and cell differentiation, from a rapid, low specificity response, a trademark of the innate immune system, to a relatively slow, highly specific response which is associated with the adaptive immune system.

1.2.1 Avian B cell development

As the BoF of Fabricius is the main organ for avian B cell development, the development of B cells can be divided into prebursal (Lebacq, A.M. and Ritter, M.A. 1979), bursal (Funk, P.E. and Palmer, J.L. 2003) and post bursal stages (Paramithiotis, E. and Ratcliffe, M.J. 1994b). Pre B cells originate from the dorsal aorta and yolk sac (Reynaud, C.A. et al. 1992). These cells are committed to the B cells lineage, as they express the B cell specific surface antigen chicken B6 (also known as Bu-1) (Houssaint, E. et al. 1991). The first heavy chain rearrangements can be detected as early as ED5 (Reynaud, C.A. et al. 1992). In chickens, this process is not a major contributor for Ig diversification (Ratcliffe, M.J. and Jacobsen, K.A. 1994). Light chain rearrangements coincide with those of the heavy chain (as opposed to mammals, where these are two separate events) (Ratcliffe, M.J. 2006). By ED15 all the gene rearrangements are complete and cells with fully rearranged genes can be found in the BoF, blood, spleen and thymus (Arakawa, H. and Buerstedde, J.M. 2004). However, continued B cell development is detectable only in the BoF, and the populations in other organs decrease (Arakawa, H. and Buerstedde, J.M. 2004). By ED10 the BoF contains roughly 10,000 follicles which are seeded by pre B cells between that time point and ED15 (Pink, J.R. et al. 1985).

The migration to the BoF is thought to be dependent upon the expression of CXC motif chemokine receptor 4 (CXCR4) by the prebursal B cells, while the BoF expresses CXC motif ligand 2 (CXCL2) as its counterpart (Meyer, R.K. et al. 1959). Another molecule that is expressed by the prebursal B cells is sialyl Lewis x (Masteller, E.L. et al. 1995b) which has a role in the cells populating the BoF. While committed B cells migrate to the BoF, only those with a functional B cell receptor which includes surface Ig (sIg)M as well as the signal transduction peptides Igα and Igβ will continue to proliferate in the follicles (Pike, K.A. et al. 2004). In fact, it was discovered that the signal transduction capabilities are necessary and sufficient in order for the cell to proliferate (Pike, K.A. and Ratcliffe, M.J. 2005).

Repeated rounds of gene conversion (Arakawa, H. et al. 2002), the main mechanism in chickens for Ig diversification, begins to occur (Mansikka, A. et al. 1990). From this moment and until ED19, B cells begin to colonize and exponentially proliferate in the bursal buds (Lydyard, P.M. et al. 1976). By ED19, 90% of bursal cells express sIg (Lydyard, P.M. et al. 1976). The end of this stage is marked by a shift in expression from sialyl Lewis x to Lewis x on the B cell surface; this shift is dependent on the bursal environment (Masteller, E.L. et al. 1995a). Post hatch, there is a redistribution of the bursal B cells between the medulla and the newly formed cortex, and while the B cells that remain in the medulla slow their proliferation rate, the B cells in the cortex of the follicles continue to divide rapidly (Reynolds, J.D. 1987). Post hatch, roughly 95% of the cells in the BoF lose their surface IgM expression and undergo apoptosis (Paramithiotis, E. et al. 1995). While it is unclear why this happens, as all B cells proliferating in the BoF have a functionally rearranged light and heavy chains, it is possible that the combination of the two is not always functional, which may lead to these low survival rates (Motyka, B. and Reynolds, J.D. 1991).

Around hatching, surface IgM positive cells begin to leave the bursal area and enter peripheral blood and then migrate into B cell areas of spleen, thymus and cecal tonsils, where they can take part in the immune response (Cooper, M.D. et al. 1969).

1.2.2 B cells in the periphery

After they acquire the ability to emigrate from BoF, 1% of the B cells destined for the periphery leaves the BoF every hour (Lassila, O. 1989). Most, but not all of these cells originate from the bursal follicular cortex. It has been demonstrated that B cells originating from the cortex and those originating from the bursal medulla have different life spans, and it is possible that these constitute functionally distinct B cell populations (Paramithiotis, E. and Ratcliffe, M.J. 1996). Like in mammals, where B cells initially emigrate from the bone marrow to the spleen, the chicken B cells also emigrate to the spleen and are situated in B cell specific areas called periellipsoidal lymphoid sheaths situated around capillaries as well as in germinal centers (Paramithiotis, E. and Ratcliffe, M.J. 1993). These areas are anatomically less defined than in mammals (Yasuda, M. et al. 1998). B cells also emigrate to secondary lymphoid tissues such as the gut associated lymphoid tissue, Peyer's patches and the cecal tonsils (Hegde, S.N. et al. 1982) and the bronchus associated lymphoid tissue (Fagerland, J.A. and Arp, L.H. 1993). While the formation of these areas is not antigen dependent, their subsequent development is (Hegde, S.N. et al. 1982). Similar to the spleen, these areas contain a mix of T and B cells which are not as clearly defined as they are in mammals (Jeurissen, S.H. 1993). In these areas the B cells are activated in either a T dependent or independent manner.

1.3 Programmed cell death

Throughout its life, every multicellular organism is presented with instances in which it has to rid itself of specific cells. These situations present themselves not only when the cells are infected or damaged, but in general, when these cells interfere with the homeostasis of the organism (Labbe, K. and Saleh, M. 2008). There are several tightly maintained "Programmed cell death" (PCD) pathways that the organism or the cell itself can activate in order to induce cellular death. These pathways are heavily controlled, as spontaneous cell death with no control over the process can be catastrophic.

There are at least three PCD pathways (Portt, L. et al. 2011). Type II PCD, also called autophagy, is a catabolic pathway that allows the cell to sequester various cellular organelles and proteins in specialized double membrane vesicles with lysosomal activities, break them down and recycle them (Gozuacik, D. and Kimchi, A. 2007). Although mostly used for intracellular homeostasis, extensive autophagy may lead to cell death (Duprez, L. et al. 2009). Type III PCD, necrosis or oncotic cell death (Majno, G. and Joris, I. 1995), was considered up until recently to be an uncontrolled form of cellular death. However, evidence was found that this form of PCD is indeed regulated, and is even used in a developmental context (Chautan, M. et al. 1999). Type I PCD is also known as apoptosis.

1.4 Apoptosis and B cell selection process

B cells with the potential to activate a potent immune response in both an innate and adaptive manner, need to be under strict control. In order to achieve this, the cells go through a rigorous

selection process that ensures that the cells leaving the BoF are functional (Thompson, C.B. 1992) as well as not strongly responsive to the organism itself (Funk, P.E. and Palmer, J.L. 2003), a trait that could cause a severe autoimmune disease (Grimaldi, C.M. et al. 2005). The selection process has two "checkpoints" that the maturing B cell has to pass in order to leave the BoF and migrate to the periphery. The first one is a positive selection process where the cells are tested for expression of a functional pre BCR complex (van der Burg, M. et al. 2002). Pre B cells that fail this selection step go into programmed cell death (Cheng, S. et al. 2009). It is unclear which of the apoptotic pathways is utilized. Some implications were made as to the role of the first apoptotic signal receptor (FAS) and its ligand in the process, pointing at the extrinsic pathway, but this subject needs to be investigated further (Opferman, J.T. 2008). The second process is negative selection. i.e., deletion of self-reactive B cell populations (Luning Prak, E.T. et al. 2011). Both selection processes are dependent on the integrity of the B cell receptor. The selection process in the BoF is extremely rigorous. Only 5% of B cells express a functional BCR complex and are not self-reactive, where as 95% of cells go through apoptosis if they fail these checkpoints (Sayegh, C.E. et al. 1999).

1.5 The B cell receptor

The BCR, in addition to its role in B cell selection, is the link between B cells and humoral immune system. The receptor complex includes a sIg as an antigen capture moiety, and a signal transduction moiety (Chu, P.G. and Arber, D.A. 2001). The latter is made up of two non-covalently bound proteins (Igα and Igβ), which are involved in the activation of the cell once the antigen is recognized (Lee, W.Y. and Tolar, P. 2013). These two proteins both have immunoreceptor tyrosine

based activation motifs (ITAMs) able of activating a signaling cascade which will result in cell activation, proliferation and antibody secretion (Cambier, J.C. et al. 2007). It is this signaling ability (as opposed to the BCR ability to bind the antigen) that allows the cell to pass the first selection process (Wienands, J. and Engels, N. 2001).

1.6 Pattern recognition receptors

One of the ways in which the innate immune system recognizes microbial elements is a family of receptors generally called the pattern recognition receptors (PRRs) (Takeuchi, O. and Akira, S. 2001, Akira, S. 2006). There are several types of PRRs, such as C-type lectins (CTLs), which recognize carbohydrates in a calcium dependent manner (Graversen, J.H. et al. 1998). Membrane bound CTLs, like dectin, recognize their ligands which will lead to activation of innate immune cells such as dendritic cells (DCs) (Ariizumi, K. et al. 2000) and macrophages (Taylor, P.R. et al. 2002). These cells will phagocytize the bound material and/or secrete cytokines to further advance the immune reaction against the intruder (Mezger, M. et al. 2008). The mannose binding lectin, which is a secreted type of CTL, can interact with the complement system to further amplify the innate immune response (Jack, D.L. et al. 2001). Other PRRs like nucleotide-binding oligomerization domain (NOD) like receptors and retinoic acid-inducible gene like receptors (RIG) are intracellular in nature, reacting with intracellular MAMPs (Kanneganti, T.D. et al. 2007). However, the largest and most well researched family of PRRs is the TLRs. The members of this family are highly conserved receptors, which can be found either in endosomes or membrane bound (Mogensen, T.H. 2009). Thirteen TLRs were found in humans (Zarember, K.A. and Godowski, P.J. 2002), with most of them having a homolog receptor in chickens (TLRs 9, 11, 12

and 13 are absent from the chicken TLR repertoire) (Keestra, A.M. et al. 2013). Different TLRs are defined by their location and ligand. For example, TLR5, which recognizes flagellin, is a membrane bound TLR, while TLR7, a single strand RNA recognizing receptor, is found in endosomes in the cytoplasm (Jimenez-Dalmaroni, M.J. et al. 2016). TLRs are expressed on several types of immune cells such as macrophages, DCs, B cells, and specific types of T cells. Furthermore, they are also expressed on nonimmune cells such as fibroblasts and epithelial cells (Akira, S. 2006). As with other PRRs, ligand recognition by the TLR will, in most cases, lead to an innate proinflammatory response (Aderem, A. and Ulevitch, R.J. 2000).

1.6.1 Chicken TLR2

While most TLRs function as homodimeric complexes, TLR2 functions as part of a heterodimer with other TLRs (Farhat, K. et al. 2008). Each of the combinations is more sensitive to different types of TLR2 ligands. A TLR1-TLR2 combination can recognize triacetylated lipoproteins found in Gram-positive bacteria such as *Clostridium* spp., *Staphylococcus aureus* and *Mycoplasma* spp., while a TLR6-TLR2 combination recognizes diacetylated lipoproteins (a Gram-positive bacteria component) and zymosan (a fungal component) (Farhat, K. et al. 2008). TLR2 had also been known to recognize LPS in the presence of another protein, lymphocyte antigen 96 (MD2) (Matsuguchi, T. et al. 2000, Yitbarek, A. et al. 2012) and several protozoan (Wong-Baeza, I. et al. 2010) and viral (Klein Klouwenberg, P. et al. 2009) ligands. Recognition of these ligands by TLR2 will activate a Myeloid differentiation factor 88 (MyD88) mediated cascade. This activation will result in the activation of the transcription factor nuclear factor kappa B (NF-κB) (Arbibe, L. et al. 2000) and the expression of several pro inflammatory cytokines such as Interleukin (IL)-1β

(Cogswell, J.P. et al. 1994) and IL-6 (Parikh, A.A. et al. 1997), which will, in turn, cause fever, acute phase protein production (Akira, S. and Kishimoto, T. 1992), macrophage activation (Chomarat, P. et al. 2000), endothelial expression of cell adhesion molecules and lymphocyte proliferation and differentiation. It has been shown that using TLR2 ligands induces both type 1 (Th1) and type 2 (Th2) helper T cell-like responses (St Paul, M. et al. 2012b), induces anti-viral responses in chickens (Barjesteh, N. et al. 2015a), and that one of the responses to *Salmonella pullorum.* infections in chickens is upregulated TLR2 expression (Ramasamy, K.T. et al. 2014). This versatility of TLR2 based responses is the reason its ligands are used in adjuvants for vaccines such as the one against avian influenza, where adding them protected the chickens from challenge as well reduced the amount of viral shedding (Barjesteh, N. et al. 2015b). This effect was seen *in ovo* as well, where administration of TLR2 ligands conveyed protection against avian influenza (Barjesteh, N. et al. 2015a) as well as against infectious laryngotracheitis virus, a protection that was detectable post hatch as well (Thapa, S. et al. 2015). While the role of TLR2 is similar in chickens and mammals, chickens have two functional genes encoding for TLR2 – TLR2a, expressed in the cecal tonsils, liver and spleen; and TLR2b, which is more broadly distributed (Fukui, A. et al. 2001).

1.7 Major histocompatibility complex class II

The MHC II is a crucial factor in presenting antigens to T cells (van den Elsen, P.J. et al. 2003). Under normal conditions, only antigen presenting cells (APCs) express MHC class II. Those cells include DCs, macrophages and B cells. Other cells, such as thymic epithelial cells and T cells (post

activation) may also express MHCII, and expression in other cells can be induced by interferon-γ (IFN-γ) (Boss, J.M. and Jensen, P.E. 2003, van den Elsen, P.J. et al. 2003).

Expression of MHCII by APCs and presentation through this complex serves different functions. DCs take up antigen through macropinocytosis and phagocytosis and present the processed antigen to naïve $CD4^+$ T cells (ten Broeke, T. et al. 2013). As long as the proper costimulatory signals are presented as well, the T cell will differentiate into one of several types of T effector cells, determined by the cytokine profile secreted by the APC (Bailey, S.R. et al. 2014). Macrophages present antigen through the MHCII receptor to effector T cells found on site; in a manner similar to that of DCs (van den Elsen, P.J. et al. 2003). Interaction between macrophages and effector T cells will result in the activation of the macrophage to produce pro inflammatory cytokines and produce nitric oxide (Bingaman, A.W. et al. 2000). B cells also present antigen to already activated T cells, however, the antigen is first recognized by the antigen specific B cell receptor and only then it is internalized and processed (Roche, P.A. and Furuta, K. 2015). Recognition of the complex by the T cell will cause that cell to produce and secrete a cytokine profile that will activate several processes in the B cell. These include antibody class change, which will match the antibody produced to the nature of immune response needed (Hu, J. et al. 2013) as well as somatic hypermutation, which will increase the specificity of the produced antibody to the antigen (Li, Z. et al. 2004). Furthermore, these cytokines will induce B cell differentiation into memory cells and plasma cells (Ettinger, R. et al. 2005, Hu, J. et al. 2013).

1.8 Nutritional intervention

There is a clear link between nutrition and immune capabilities. It is easiest to observe while discussing malnutrition, the damage it causes to the organism in general and to the immune system specifically. However, a positive link exists between specific nutrients and immune capabilities (Fox, C.J. et al. 2005, Zhao, R. et al. 2016). Food components such as iron and vitamin D have considerable potential to reduce susceptibility to infectious diseases (Katona, P. and Katona-Apte, J. 2008). Examples in poultry include amino acids such as arginine or threonine (Kidd, M.T. et al. 2001), β-glucans (Lowry, V.K. et al. 2005) and zinc and selenium as minerals which have a wide spectrum of effects on the immune system (Stahl, J.L. et al. 1989).

The race to improve the immune capabilities of production animal such as the chicken through nutritional intervention is crucial, especially since a growing number of countries ban the use of antibiotic growth promoters, as these might have adverse effects on the animals gut microbiota (Yitbarek, A. et al. 2013), may affect nutrient absorption (Knarreborg, A. et al. 2004) and could contribute to microbial resistance to antibiotics (Dibner, J.J. and Richards, J.D. 2005). Another reason the chicken industry may benefit from immunological nutritional intervention is that the selection for rapid growth and egg production has dampened the immune response (Leshchinsky, T.V. and Klasing, K.C. 2001).

Nutritional intervention can be achieved with three main strategies: Probiotics, prebiotics and synbiotics. Probiotics are live microbes which confer health benefit to the host (Sanders, M.E. 2008) and compete with pathogens for nutrient absorption and colonization space (Deriu, E. et al. 2013). The use of these beneficial bacteria has been shown to have a direct effect on the immune

capabilities of the host (Malin, M. et al. 1996, Isolauri, E. et al. 2001). The use of *Lactobacillus fermentum* and *Saccharomyces cerevisiae*, for example, has been shown to effect TLR expression and the T cell population in the chicken intestine (Bai, S.P. et al. 2013), and *Clostridium butyricum* has an effect on cytokine production (Zhang, L. et al. 2016). Commensal bacteria can also produce nutrients that are utilized by the host such as short chain fatty acids (Topping, D.L. 1996) and vitamins such as vitamin K, and those in vitamin B family (Resta, S.C. 2009) such as B9 (Rossi, M. et al. 2011), also known as folic acid (FA). Another strategy is the use of prebiotics, materials that either benefit the commensal bacteria population of the gut or the host directly (Roberfroid, M.B. 2000). Yeast derived nucleotides (Yitbarek, A. et al. 2013) have been shown to have an effect on TLR2b expression and cytokine production. The combined administration of probiotics and prebiotics is called synbiotics, and there has been extensive research done on the subject in humans (Chang, Y.S. et al. 2016), mice (Simeoli, R. et al. 2015) and poultry (Rodriguez-Lecompte, J.C. et al. 2012, Madej, J.P. and Bednarczyk, M. 2016).

1.9 FA in nutritional intervention

FA has been investigated thoroughly as a nutritional immune modulator for several reasons. First, it is a substance that can be obtained both nutritionally as well as a metabolite of commensal bacteria such as *Bifidobacterium adolescentis* (Strozzi, G.P. and Mogna, L. 2008). Secondly, it is part of several integral pathways including DNA synthesis and methylation. It was found to be extremely important in human nutrition, especially in expecting mothers, where FA deficiency may cause neural tube defects (Grosse, S.D. and Collins, J.S. 2007) as well as problems in embryonic production of normal erythrocytes (Carmel, R. 2008) and leukocytes (Kaplan, S.S. and

Basford, R.E. 1976) in the developing embryos. FA supplementation is considered so beneficial, that Canadian and US lawmakers mandated the FA fortification of cereal grain products (Tactacan, G.B. et al. 2010). This led to the exploration of egg enrichment with FA through the hen's diet (Seyoum, E. and Selhub, J. 1998). Egg enrichment with FA lead to increased egg folate concentrations, with an enriched egg containing approximately 10% of the recommended dietary allowance for adults (Tactacan, G.B. et al. 2010). However, beyond a certain point FA addition to the hens diet leads to saturation. Addition of more than 3.6 μg/kg FA to the diet does not result in higher concentrations of FA in the egg, (House, J.D. et al. 2002). There are other factors found to be altered through FA addition to poultry diet, including immunological factors such as alterations to the T cell population (Wintergerst, E.S. et al. 2007) and expression of the proinflammatory cytokine IL-1β (Munyaka, P.M. et al. 2012).

1.10 FA transport

There are two major folate transporters. The proton coupled folate carrier (PCFT) mediates intestinal absorption of FA. Its optimal activity is at low pH values found in the upper part of the intestinal tract (Visentin, M. et al. 2014). The reduced folate carrier (RFC) is a neutral pH membrane transporter of FA (Sierra, E.E. et al. 1997). It is ubiquitously expressed throughout the body (Whetstine, J.R. et al. 2002) including in B cells (Baslund, B. et al. 2008) and the BoF (Jing, M. et al. 2009). It is crucial for proper embryonic development (Zhao, R. et al. 2001), and is considered to be the major folate transport system in mammals (Matherly, L.H. et al. 2007). The RFC has a much higher affinity for 5-methyltetrahydrofolate, the major form of reduced FA in the blood, than to unreduced FA by several orders of magnitude (Zhao, R. et al. 2001). Interestingly,

the expression of both receptors is downregulated either at the mRNA level, post transcriptional or translational level or both, when exposed to their ligand (Ashokkumar, B. et al. 2007, Tactacan, G.B. et al. 2012). Conversely, Under FA deficiency conditions both receptor types are over expressed (Thakur, S. and Kaur, J. 2015). This negative association is caused by reduced promoter activity, reduced messenger RNA (mRNA) levels (Ashokkumar, B. et al. 2007) and sequestration of RFC protein in the endoplasmatic reticulum (Hou, Z. et al. 2014).

1.11 Epigenetics

Epigenetic is defined as changes in gene expression, some of which are heritable, with no change in the DNA sequence (Chuang, J.C. and Jones, P.A. 2007). A lack of dependence on the genomic sequence, and their hereditary nature sets epigenetic mechanisms apart from gene expression control mechanisms that rely on transcription factors, enhancers and promoters, the latter having a transient effect on gene expression, and rely on the sequence of the genetic material (Tammen, S.A. et al. 2013).

1.11.1 Epigenetic Factors

The DNA double strand which make up the genetic data in each cell is wrapped around specialized protein structures called histones, which are made up of four sets of homodimers. A unit made up of a histone and the DNA wrapped around it is called a nucleosome (Rothbart, S.B. and Strahl, B.D. 2014). These units make up the chromatin. This form of packaging makes it possible for vast amounts of genetic material to be stored inside the nucleus in chromosomal form (Tammen, S.A. et al. 2013). Each one of these structures – the DNA, the histone and the chromatin,

can go through several modifications that will make the genetic material more or less accessible for expression (Rothbart, S.B. and Strahl, B.D. 2014). The histone, for example, has its N terminal end protruding from outside of the globular structure. This part of the protein can go through several types of modifications such as methylation and acetylation (Allfrey, V.G. et al. 1964). Methylating the histone would make the nucleosome structure bind more firmly together into a structure called Heterochromatin, making it difficult or even impossible to transcribe the genes present in that structure, depending on the amount of methylation done to the site (mono-, di- or trimethylation). Acetylating the histone, on the other hand, has the opposite effect. Due to the change in the electrical charge of the histone post acetylation the chromatin will become less tightly wrapped and more available for the transcription process (Euchromatin), thus allowing access to the genes wrapped around those histones (Allfrey, V.G. et al. 1964). Other histone modifications include phosphorylation, ubiquitination and biotinylation (Tammen, S.A. et al. 2013). The effect of each modification on the structure of the histone and the availability of the genetic code is called the histone code (Rothbart, S.B. and Strahl, B.D. 2014).

1.12 DNA Methylation

Like histones, the DNA can also be methylated. Most methylation occurs on the 5’ carbon on cytosines that are part of a cytosine-guanine (CpG) dinucleotide, although some methylation has been shown to occur on other nucleotides. It is an enzymatic reaction, carried out by the enzyme family DNA methyltransferases (DNMT) by using S-adenosylmethionine (SAM) as a methyl donor (Crider, K.S. et al. 2012).

The methyl donor used in the process of methylation, SAM, is a derivative of FA. When FA is absorbed in the intestine it is reduced enzymatically in the enterocytes into tetrahydrofolate (THF). THF is converted into 5-methyl tetrahydrofolate (5-mTHF) in the liver and is then released into the circulation (Hebert, K. et al. 2005). Other derivatives of this substance are used in DNA synthesis, while 5-mTHF is used to convert homocysteine to methionine. Methionine is then reduced to SAM, which is used by the DNMT enzyme family (Crider, K.S. et al. 2012). While FA can be obtained dietarily, through foods like beans, asparagus and avocados (Wald, D.S. et al. 2001), some components of the gut flora, like the *Bifidobacterium spp.* can produce this vitamin, making them a model probiotic species (Pompei, A. et al. 2007). Other dietary methyl donors include betaine, choline, methionine, vitamin B12 and vitamin B6. These donors all converge into the homocysteine-methionine pathway described above (Olthof, M.R. et al. 2005).

Epigenetic regulation in general, and specifically DNA methylation, is not a transient process. These control mechanisms are in charge of determining the gene expression profile of the cell type. i.e., which genes will be available for transcription at every stage of the cells life (Ghoshal, K. and Bai, S. 2007). Furthermore, that profile is hereditary, ensuring the daughter cells, being the same type as the mother cell, will have the same genes available for transcription. There are two types of DNA methylation processes that are separated by their function and the enzymes that are used. DNMT3a and DNMT3b carry out nonhereditary, or "de-novo methylation". This process establishes methylation patterns during early development as well as in maternally imprinted genes in oocytes (Gowher, H. and Jeltsch, A. 2001). DNMT3a and DNMT3b have the ability to methylate cytosines regardless of other methylated sites on the DNA strand. This process has been shown to work in differentiated somatic cells as well, but at a slower rate (Gowher, H. and Jeltsch,

A. 2001, Hata, K. et al. 2002). Conversely, DNMT1 is responsible for "maintenance methylation". This type of methylation is possible due to the semi conservative model of DNA replication in eukaryotic cells. In this model, each daughter strand has one strand for the mother cell and one new strand. DNMT1 identifies locations on the mother strand where the cytosines are methylated, and methylates the corresponding cytosines on the new strand (Ghoshal, K. and Bai, S. 2007). In any case, methylation of a group or single CpG dinucleotides acts as steric interference, inhibiting transcription factor binding to the region.

Another way in which DNA methylation inhibits gene expression is the recruitment of methylated DNA binding proteins such as Methyl CpG binding protein (MeCP2), methylated DNA binding protein (MBD)1 and MBD2 (Russ, B.E. et al. 2013). These protein complexes recruit histone methyltransferases and histone deacetylases, which have the ability to "close" the chromatin, forming heterochromatin. In this way, these two major mechanics of epigenetic control interact with each other to ensure complete opening or closing of the target loci (Russ, B.E. et al. 2013).

In stem cells and cells that have not yet fully differentiated, methylated sites begin to appear on the DNA sequence (due to DNMT 3a and DNMT 3b) (Okano, M. et al. 1999). Through the differentiation of the cell areas in the genome containing genes that are irrelevant for that cells function are placed in a heterochromatin formation (Li, E. 2002). The end result is a gene expression profile specific for that type of cell. A fully differentiated cell that is part of a specific system or organ has a gene expression profile that best suits the function of this cell. The profile will be passed on to the progeny of the cell because of the function of DNMT1 (Bestor, T.H. 2000).

1.13 Apoptosis and DNA methylation

The relationship between apoptosis and DNA methylation is a two way street. As DNA methylation in general and the function of DNMT1 specifically are essential for the proper development of the organism, lack of a proper methylation profile may lead to apoptosis of the cell (Jackson-Grusby, L. et al. 2001). This is also apparent in neoplastic cells, where inactivation of DNMT1 also results in programmed cell death (Chen, T. et al. 2007). Hypermethylation of promoter regions has a crucial role in cell differentiation and embryogenesis (Geiman, T.M. and Muegge, K. 2010). However, it was found that in certain types of recurring cancers the promoter region of caspase 8 is hypermethylated, inhibiting apoptosis of cancerous cells, which contributes to their progression (Martinez, R. et al. 2007). Hypermethylation can also interfere with the intrinsic apoptotic pathway, as has been shown for the proapoptotic Bcl-2-associated X protein (Bax) gene (Hervouet, E. et al. 2013).

1.14 TLR2b and DNA methylation

The relationship between TLR2 promoter methylation and expression varies between cell types. For example, in bronchial epithelial cells hypermethylation of the promoter region resulted in increased sensitivity to TLR2 ligand (Shuto, T. et al. 2006), while in endothelial cells, mRNA levels were not affected by changes in the TLR2 promoter region methylation status. Hypermethylating the promoter region of TLR2 in gingival epithelial cells resulted in down regulation of the mRNA and an increased susceptibility to disease (Benakanakere, M. et al. 2015). In chickens, treatment with 5-Aza-2′-deoxycytidine, a demethylation agent, did not have any effect on the expression of TLR2b, while the expression of TLR2a was upregulated (Gou, Z. et al. 2012).

1.15 The BCR signal transfer moiety and DNA methylation

For the mammalian B cell to express Igα, a complex made up of the paired box protein (Pax)5 and the E26 transformation-specific proteins (ETs) is required to act as a transcription factor for the CD79a gene (Maier, H. et al. 2003b). The formation of this complex is only possible when the CD79a promoter site is hypomethylated (Maier, H. et al. 2003a), which is its status in B lineage cells (Maier, H. et al. 2003a). In other cells a single CpG dinucleotide in the promoter region of CD79a is methylated, which interferes with the formation of the Pax5-Ets complex (Maier, H. et al. 2003a). Furthermore, there is some evidence that mammalian Igβ is also under DNA methylation control (Malone, C.S. et al. 2006). Examination of epigenetic controls placed on the Igβ promoter region in chicken cells revealed that in tissues where the gene is not transcribed the promoter region is highly methylated (Itaya, K. et al. 2011, Minbuta, T. and Ono, M. 2011). However, others described the Igβ gene in chicken B cells to be epigenetically controlled through histone modification, with no changes to the methylation status of the promoter region even after the formation of euchromatin through histone acetylation (Shimada, N. et al. 2006). Both proteins increase the efficiency of another B cell trait – antigen presentation through MHC II (Bonnerot, C. et al. 1995).

1.16 Major histocompatibility complex class II and DNA methylation

While it is true that some cells can be induced to express MHC class II, some cells will not express this complex even when exposed to interferon-γ. These include sperm (Kuhlmann, D. et al. 1986), oocytes (Dohr, G.A. et al. 1987) and trophoblasts (Redman, C.W. et al. 1984). Thus, these types of cells can evade maternal immune recognition, allowing for a successful pregnancy (van den

Elsen, P.J. et al. 2003). This lack of expression of MHC is not due to absence of the transcription factors but rather due to the methylation of the promoter regions of the genes involved. While there are several factors necessary for MHC II expression, there is one crucial factor that is needed. This protein is constitutively expressed in antigen presenting cells, however in cells that do not express MHCII, the promoter for class II transactivator (CIITA) is highly methylated (van den Elsen, P.J. et al. 2003). In this way, MHC II expression is regulated through indirect DNA methylation. Treatment of these cells with 5' deoxyazacytidine, a known DNA demethylator, resulted in both CIITA and MHC II expression following exposure to IFN-γ (van den Elsen, P.J. et al. 2000). The hypermethylation of the CIITA promoter is also apparent in leukemic T cells, where it effectively inhibits MHC II expression (van den Elsen, P.J. et al. 2003).

The HLA class II gene family expression pattern exhibit all three possible relationships with promoter methylation. While some genes are downregulated when their promoter is hypermethylated, others are upregulated and in a third case there is no correlation between promoter methylation and gene expression (Levine, F. and Pious, D. 1985). To the best of our knowledge, no papers examining the effect of promoter methylation on MHCII expression in chickens have been published.

As B cell development is sensitive to methyl supply due to their high proliferation rate, characterization and evaluation of methylation patterns in TCR, BCR and MHC II expression and their association with apoptosis induction are warranted. Specifically, the need to determine whether nutritional bioactive folate is able to influence methylation patterns in the genes involved in the formation of TCR, BCR and MHC II affecting immune and health status.

1.17 Hypothesis

Inclusion of FA influences methylation patterns in B cell precursor development in chicken embryos affecting innate and acquired immune responses in B cells through TLR2, BCR, and MHCII gene expression status.

1.18 Research objectives

1. To study epigenetic changes in the proximal promoter region associated with TLR2b, BCR intracellular signaling protein Igβ, and MHCII genes in B cell precursors and to identify genes under methylation regulation. In order to achieve this objective the proximal promoter region (200 bp upstream of the start codon) will be sequenced to determine the number and location of CpG dinucleotides.

2. To determine whether inclusion of a methyl donor will affect DNA methylation in the proximal promoter region of TLR2b, Igβ, and MHCII genes. In order to achieve this objective cells will be incubated with a methyl donor. The DNA from these cells will be treated with bisulfite and the promoter region amplified and sequenced to determine which of the CpGs in this region are methylated and which are not.

3. To determine whether epigenetic programming by DNA methylation regulates gene expression. In order to achieve this objective RNA from the treated cells will be used to determine changes in TLR2b, Igβ and MHCII β chain expression following treatment with a methyl donor.

The aim of the thesis is to characterize the epigenetic mechanism of DNA methylation, as playing a role in embryonic B cells precursors and in expression of cell sIg and subsequent B cell proliferation in bursal follicles. DNA modification involves methylation of cytosine at the carbon-5 position (5-methyl cytosine; 5mC) in CpG dinucleotides. Furthermore, chromatin remodeling is mediated by DNA methylation and demethylation. FA will be used as a methyl donor model to evaluate the epigenetic changes in chicken embryo B cells and the impact that folate has on expression of TLR-2, Igβ (BCR), and MHC II genes.

2. General Materials and methods

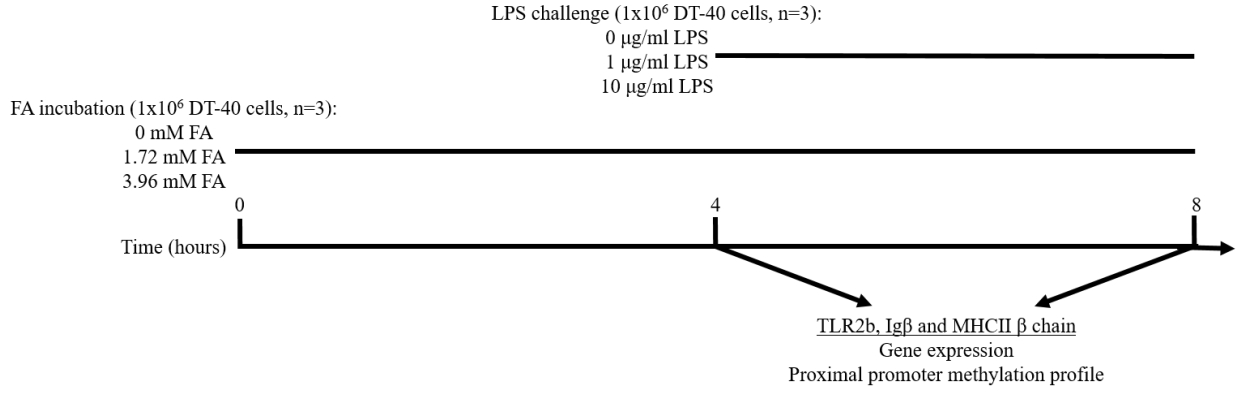
2.1 Ethical considerations

All the experimental procedures that chicken embryos were subjected to are consistent with the guidelines set out by the Canadian Council on Animal Care as approved by the University of Prince Edward Island Animal Care Committee.

2.2 Experimental designs for experiments conducted with DT-40 cell line

The first series of experiments was conducted on samples containing $1x10^6$ cells from a cell line derived from an avian leukosis virus induced bursal lymphoma (DT-40) cells. The cell culture was pipetted gently before distributing the cells for each treatment to achieve completely randomized sampling. The design of the experiments is presented in Figure 2.1

Figure 2.1 DT-40 cell line experiments design flow chart



2.2.1 Association between FA and proximal promoter methylation of TLR2b, Igβ and MHCII β chain

FA was tested at three levels: 0 mM FA, 1.72 mM FA and 3.96 mM FA. Each treatment was repeated three times independently for both 4 and 8 hours of incubation for a total of 9 samples for each gene at each incubation time.

2.2.2 Association between percent of promoter methylation and mRNA levels of TLR2b, Igβ and MHCII β chain

Samples from all three levels of FA treatment groups (0 mM FA, 1.72 mM FA and 3.96 mM FA) were analyzed, for a total of 9 samples for each gene at each incubation time.

2.2.3 The Effect of incubation time and FA on mRNA levels of TLR2b, Igβ and MHCII β chain

FA concentration and incubation time are the two main factors in this design. FA was tested at three levels: 0 mM FA, 1.72 mM FA and 3.96 mM FA. Incubation time was tested at two levels: 4 hours and 8 hours. Each treatment was repeated three times for a total of 9 samples for each gene at each incubation time.

2.2.4 The effect of LPS and FA on the mRNA levels of TLR2b, Igβ and MHCII β chain

FA and LPS are the two main factors in this design. FA was tested at three levels: 0 mM FA, 1.72 mM FA and 3.96 mM FA. LPS was tested at three levels: 0 μg/ml, 1 μg/ml and 10 μg/ml. Each treatment was repeated three times for a total of 27 samples for each gene.

2.2.5 The effect of FA on the mRNA levels of TLR2b, Igβ and MHCII β

Each FA treatment was repeated three times for either 4 or 8 hours of incubation and the response was analyzed separately for each incubation time. The 0 mM FA treatment acted as a control group for each gene (n=3).

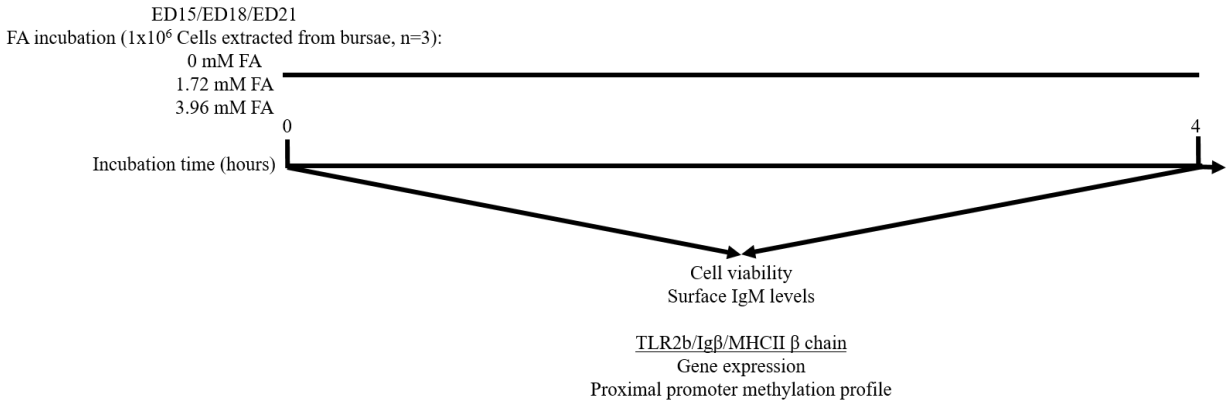
2.2.6 The effect of FA on mRNA levels after treatment with LPS

Each FA treatment was repeated three times on samples containing cells treated with either 1 or 10 μg/ml LPS and the response was analyzed separately for each LPS treatment. The 0 mM FA treatment acted as a control group for each gene (n=3).

2.3 Experimental designs for experiments conducted with cells extracted from embryonic BoF

The second series of experiments was conducted on samples containing $1x10^6$ cells harvested from embryonic BoF. Fertile eggs used to harvest the cells were randomly selected and the cell culture was pipetted gently before distributing the cells for each treatment to achieve completely randomized sampling. The design of the experiments is presented in Figure 2.2

Figure 2.2 Bursal B cells experiments design flow chart



2.3.1 The effect of ED and incubation time on the percent of live cells

ED and incubation time are the two main factors in this design. ED was tested at three levels: ED15, ED18 and ED21. Incubation time was tested at two levels: 0 hours and 4 hours. Each treatment was repeated three times for a total of 18 samples.

2.3.2 The effect of ED and incubation time on the proportions of the cell populations expressing low, medium and high levels of surface IgM

ED and incubation time are the two main factors in this design. ED was tested at three levels: ED15, ED18 and ED21. Incubation time was tested at two levels: 0 hours and 4 hours. Each treatment was repeated three times for a total of 18 samples.

2.3.3 The effect of ED and incubation time on the proportions of live and dead cell populations expressing low, medium and high levels of surface IgM

ED and incubation time are the two main factors in this design. ED was tested at three levels: ED15, ED18 and ED21. Incubation time was tested at two levels: 0 hours and 4 hours. Each treatment was repeated three times for a total of 18 samples.

2.3.4 The effect of ED and FA on the percent of live cells population, proportions of sIgM expression and double stained cell populations

ED and FA are the two main factors in this design. ED was tested at three levels: ED15, ED18 and ED21. FA was tested at three levels: 0 mM FA, 1.72 mM FA and 3.96 mM FA. Each treatment was repeated three times for a total of 27 samples. The same model was used to examine the effect of ED and FA on the population proportions of sIgM expression and double stained cell populations.

2.3.5 The effect of incubation time and FA on mRNA levels of the reduced folate carrier (RFC), TLR2b, Igβ and MHCII β mRNA levels

The same model was used to examine the effect of FA on mRNA levels. Each FA treatment was repeated three times on cells harvested from embryos at ED15, D18 and ED21, and the response was analyzed separately for each age. For each gene the 0 mM FA treatment acted as a control group (n=3).

2.3.6 Association between FA and proximal promoter methylation of TLR2b, Igβ and MHCII β chain and association between proximal promoter methylation and expression of these genes.

FA was tested at three levels: 0 mM FA, 1.72 mM FA and 3.96 mM FA. Each treatment was repeated three times independently on cells harvested from embryo at ED15, ED18 and ED21 for a total of 9 samples for each gene at each embryonic age.

To examine the relation between percent of promoter methylation and mRNA levels of TLR2b, Igβ and MHCII β chain a total of 9 samples were tested for each gene at each incubation time.

2.4 DT-40 Cell culture

Chicken B cell derived DT-40 cell line cultures (American type culture collection (ATCC), Manassas, VA, USA) were grown overnight and counted on a Reichert Bright line Hemocytometer (Hausser Scientific, Horsham, PA, USA) using trypan blue (Sigma Aldrich, Oakville, ON, Canada) exclusion test to establish viability. Cells were diluted with growth medium consisting of Dulbecco's Modified Eagle's Medium (DMEM) (ATCC, Manassas, Virginia, USA) supplemented with 10% fetal bovine serum, 5% chicken serum, 55 μM 2-mercaptoethanol and 1% penicillin/streptomycin to a concentration of $1x10^6$ cells/ml, and 1 ml was seeded in each well of a 48-well plate (Corning Inc., Corning, NY, USA). The plate was incubated overnight in an MCO-19AIC incubator (Panasonic, Wood Dale, IL, USA) at 37°C under 5% CO_2 with the described growth media as per manufacturer's instructions. Cells were then treated with 0, 1.72 or 3.96 mM FA (6R, S)-5-formyl-5, 6, 7, 8-tetrahydrofolic acid, calcium salt (FA) (Schircks Laboratories, Jona,

Switzerland). 1.72 mM FA is the concentration found in the egg yolk of a hen fed a normal NRC approved diet and 3.96 mM FA is the maximal amount found in the yolk taken from a hen fed a diet supplemented with FA (House, J.D. et al. 2002). Cells were challenged with 0, 1 or 10 μg/ml of *Escherichia coli* O26:B6 LPS (Sigma Aldrich, Oakville, ON, Canada). These LPS concentrations have been used before to challenge DT-40 cells (Hawkins, B.J. et al. 2010, Kim, W.H. et al. 2014)

2.5 Egg incubation

Sixty fertilized eggs from Ross broiler breeder layers were supplied by Atlantic Poultry Inc. (Atlantic Poultry Inc., Port Williams, NS, Canada) and incubated in a Humidaire egg incubator until needed. Temperature was kept at 37°C and relative humidity was kept at 55%, both were monitored daily by using Traceable Hygrometer (Thermo Fisher Scientific, Waltham, MA, USA). The incubator was set to rotate every 6 hours. Egg candling was performed daily starting from at 7^{th} day of incubation to observe angiogenesis and determine which eggs were fertile.

2.6 BoF extraction from embryos

In a biological safety cabinet embryos were removed from the egg and euthanized by cervical dislocation which was confirmed by palpation. The abdomen of the embryo was opened using scissors and the skin above the joint connecting the femur to the ilium was cut to allow for freedom of movement. All internal organs were removed and the colon was used as a guide to the BoF location. Sterile gauze was applied as needed to clear fluids and obstructions. BoF was removed whole and placed immediately in sterile water, to allow for excess cells outside the BoF to go

through a lysis process. The BoF remained in water until the next BoF was ready to be excised. The BoF was then transferred to growth medium ATCC DMEM supplemented with 10% fetal bovine serum, 10% chicken serum, 55 μM 2-mercaptoethanol and 1% penicillin/streptomycin.

2.7 BoF Cells extraction

Pools of BoF were placed in a glass homogenizer together with 2 ml of the above described growth media. The number of BoF from each age needed for the experiments varies from age to age. Thirty BoF were extracted from ED15 embryos, 10 from ED18 embryos and 4 from ED21 embryos. The BoF were gently homogenized using a loose fitting glass homogenizer (Compton, M.M. and Waldrip, H.M. 1998). The homogenate was then passed through a 40 μM filter (Thermo Fisher Scientific, Waltham, MA, USA) and the filtrate retained. The material that did not pass the sieve was incubated for 10 minutes at 37°C with 0.5% trypsin in 1xsterile phosphate buffered saline (PBS) and 1% penicillin/streptomycin. Growth media was added to counter the effect of the trypsin and the material filtered using a 40 μM filter. Both filtrations were filtered with 100 μM filter (Thermo Fisher Scientific, Waltham, MA, USA), and the filtrate centrifuged for 5 minutes at 400 G. The supernatant was discarded and the cell pellet re-suspended with fresh growth media. Cells were counted using a Reichert Bright line Hemocytometer and trypan blue exclusion test was used to determine viability.

2.8 Total RNA Extraction and Reverse Transcription

Total ribonucleic acid (RNA) was extracted from the DT-40 cell cultures using Ambion TRIzol® Reagent (Thermo Fisher Scientific, Waltham, MA, USA) following the manufacturer's

instructions. The ethanol precipitate was cleaned using the Qiagen RNeasy minikit (Qiagen, Toronto, ON, Canada) according to the manufacturer's instructions. Genomic deoxyribonucleic acid (DNA) was extracted from DT-40 cell culture using Qiagen DNeasy blood and tissue kit (Qiagen, Toronto, ON, Canada). RNA, DNA and protein were extracted from BoF cells using a TRIzol®LS Reagent (Thermo Fisher Scientific, Waltham, MA, USA) according to manufacturer's instructions. Concentration of RNA and DNA of all samples was determined at an optical density of 260 nm and RNA purity was verified by evaluating the ratio of optical density of 260 nm to optical density of 280 nm using the ND-1000 Nanodrop (Thermo Fisher Scientific, Waltham, MA, USA). Reverse transcription of RNA samples was done using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Mississauga, ON, Canada) following the manufacturer's protocol in a Techne TC-412 thermocycler (Bibby Scientific, Burlington, NJ, USA). The complementary DNA (cDNA) was stored at –20°C.

2.9 Cloning

Primers were designed to flank the annealing sites of the real time detection assay primers of TLR2b, Igβ and RFC. After the reverse transcription process a sample was run with these primers and the product cleaned using QIAquick polymerase chain reaction (PCR) purification kit. The cleaned PCR product was then used in a cloning procedure using Topo TA cloning kit (Thermo Fisher Scientific, Waltham, MA, USA) as per manufacturer's instructions. Cloning primers, the annealing temperatures used and the expected amplicons are presented in Table 2.1. The plasmid and primer set for the house keeping gene β-actin was supplied by Dr. Rodriguez's lab, and the plasmid for MHCII β chain was a kind gift courtesy of Dr. Sharif (Ontario Veterinary College,

University of Guelph, Guelph, ON, Canada). It arrived on Whatman paper that was soaked for 30 minutes with 50 µl Tris(hydroxymethyl)aminomethane-Ethylenediaminetetraacetic acid (TE) buffer. This was used to transform bacteria as recommended by the cloning kit manufacturer.

Selective agar plates were made by dissolving 1% bacto tryptone, 0.5% bacto yeast extract and 1% NaCl in water. pH was adjusted to 7.5 with 5N NaOH. Granulated agar (1.5%) was added and the mix autoclaved (All materials purchased from Thermo Fisher Scientific, Waltham, MA, USA). After cooling to 55°C ampicillin was added to a final concentration of 0.1 mg/ml and the broth was poured into sterile petri dishes (Thermo Fisher Scientific, Waltham, MA, USA) and then left at room temperature to solidify. Plates were stored at 4°C until needed.

The transformed bacteria were plated and incubated overnight at 37°C. White colonies were selected and plated on a master plate, which was incubated under the same conditions. Colonies were then tested using a direct colony PCR with the primers designed for the real time assay (Table 2.2). Briefly, a small portion of the colony was picked with a sterile loop and placed in 10 µl of water inside a 1.5 ml Eppendorf tube. The tube caps where sealed with parafilm to avoid evaporation, boiled for 5 minutes and placed directly on ice. A 1 µl sample was used in the reaction. Colonies which were positive for the insert were placed on a new plate.

2.10 Miniprep procedure

Broth (2xYT) was made using 1.6% Bacto Tryptone, 1% Bacto Yeast Extract and 0.5 % NaCl in water, adjusted to pH7 with 5N NaOH and autoclaved. Three positive colonies were incubated overnight in 5 ml of 2xYT broth with 50 µg/ml ampicillin at 37°C while lightly shaken. The

cultures then went through and alkaline lysis miniprep procedure. Briefly, the culture was centrifuged and the supernatant discarded. A lysis buffer (50mM glucose, 10 mM Ethylenediaminetetraacetic acid (EDTA), 25 mM Tris pH8) was added to the pellet followed by an alkaline solution (0.2N NaOH + 1% Sodium dodecyl sulfate (SDS)) and 3M Potassium 5M acetate solution. Plasmid DNA was then precipitated using 95% Ethanol. The pellet was re suspended using TE buffer with 20 µg/ml RNase A. Concentration and purity of the plasmid DNA was measured using the Nanodrop (Thermo Fisher Scientific, Waltham, MA, USA) as described.

2.11 Calibration curves and efficiency values for quantitative Real-Time PCR assays

For each gene of interest, the miniprep product was serially diluted in $\log_{10}$ increments and the dilutions used to optimize the assays in order to have an efficiency curve between 90% and 110%. Each dilution was run 3 times, together with three negative controls, where the sample was replaced with water. Regression analyses of mean cycle threshold (Ct) values of six sequential dilutions were used to generate a standard curve. The slope of the curve was used to calculate the efficiency of the reaction as: $E = -1+10^{(-1/\text{slope})}$. This process was repeated three times throughout the project to ensure that the real time PCR systems maintain their ability to work in an acceptable efficiency.

2.12 Quantitative Real-Time PCR

Quantitative real-time PCR was performed in triplicate for each sample for TLR2b, Igβ, MHCII β chain and β-actin expression using the "CFX connect" real time system (Bio-Rad, Mississauga, ON, Canada) on a 96-well plate with 10 µl of total reaction volume containing 4 µl sample, 5 µl

"SsoAdvanced™Universal SYBR® Green Supermix" (Bio-Rad, Mississauga, ON, Canada) and 0.5 µl of each primer (20 µM). Genebank database (National Center for Biotechnology Information (NCBI), Bethesda, MD, USA) sequences corresponding to each quantified gene were used to design primers (Table 2.2), which were obtained from Mobix laboratories (Mobix laboratories, Hamilton, ON, Canada). The thermal cycling protocol consisted of an initial denaturation at 95°C for 3 min, followed by amplification for 40 cycles at 95°C for 10 seconds, annealing as described in Table 2.2 for each of the primer pairs, and extension at 72°C for 30 seconds. Expected product sizes are described in Table 2.2. A sample containing 100 ng/µl plasmid bearing an insert for the corresponding gene was used as a positive control, and water was used as a non-template control.

2.13 Bisulfite modification

Qiagen's EpiTect kit for bisulfite conversion (Qiagen, Toronto, ON, Canada) was used according to manufacturer's instructions. Briefly, 2 µg DNA in a 20 µl volume was used for each reaction which was mixed with 85 µl bisulfite mix and 35 µl DNA protect buffer. Bisulfite conversion was performed in a Techne TC-412 thermocycler as follows: 99°C for 5 minutes (min), 60°C for 25 min, 99°C for 5 min, 60°C for 85 min, 99°C for 5 min, 60°C for 175 min and 20°C indefinitely. The bisulfite-treated DNA was recovered by EpiTect spin column.

2.14 Bisulfite Sequencing PCR

Primers for the proximal promoter region, 200 bp directly upstream of the translation start site of TLR2b, Igβ and MHCII β chain genes were designed for bisulfite treated and untreated template

and purchased from Mobix Laboratories (Mobix Laboratories, Hamilton, ON, Canada). Primers, accession numbers and running conditions are presented in Table 2.3.

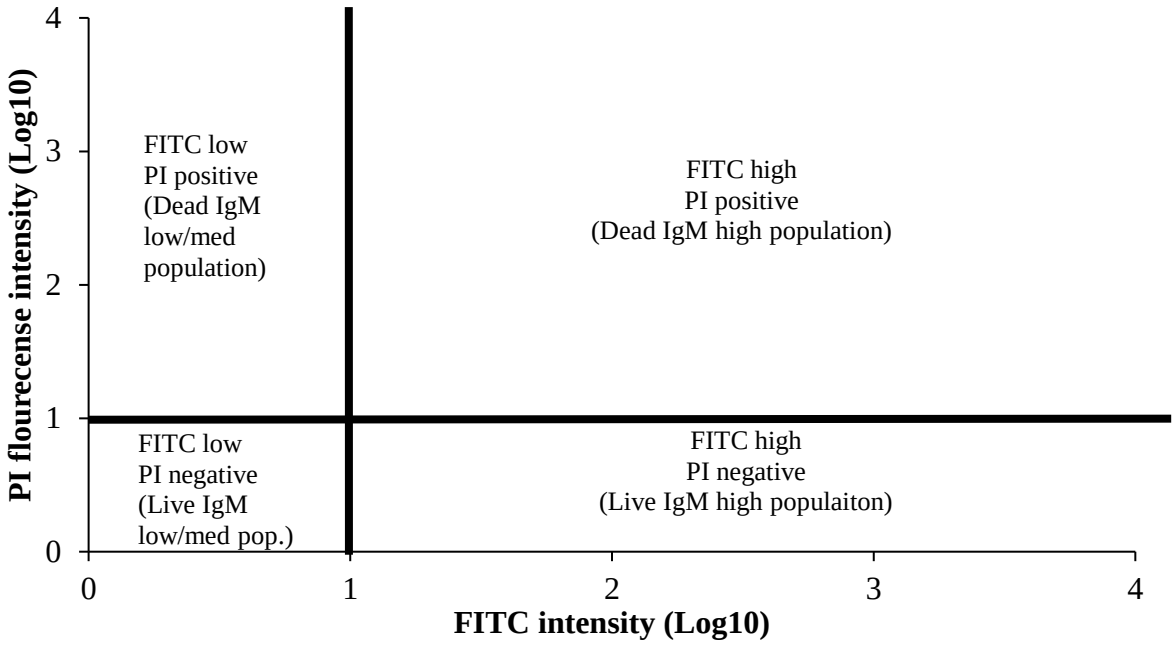
Three PCR products for each gene of interest were cleaned using QIAquick PCR purification kit (Qiagen, Toronto, ON, Canada), the sample was sent to Mobix laboratories for sequencing using ABI BigDye terminator chemistry. The process is fully automated from sample loading to data analysis. The sequence was compared to the untreated promoter region sequences found in the NCBI database, and analyzed for methylated and unmethylated CpG dinucleotides using the Biq analyzer software (Max-Planck-Institute for Informatics, Saarland University, Saarbrücken, Germany). The software excludes any sequences with less than 90% homology to the published promoter regions.

2.15 Flow cytometry

Cells were harvested from the BoF and placed in Falcon 5 ml polystyrene round bottom tubes. Cells were then washed with 2 ml 1xPBS+1% bovine serum albumin (BSA) twice and re-suspended with 200 µl 1xPBS+1% BSA. Control group of cells included unstained cells, an isotype control using Mouse IgG2B-FITC (clone A-1), mouse anti chicken IgM-FITC (clone M-1) and Propidium iodide (PI) (Sigma Aldrich, Oakville, ON, Canada). Samples were double stained with mouse anti chicken IgM-FITC (clone M-1) and Propidium iodide. All antibodies were purchased from Southern Biotech (Southern Biotech, Birmingham, Alabama, USA). Samples were incubated with the corresponding antibodies (1 µg clone M-1, 10 µl isotype antibody or 2 µg PI for 30 minutes in the dark. Cells were washed with 0.5 ml 1xPBS+1% BSA. Flow cytometry was done using BD FACSCalibur flow cytometry machine (BD Biosciences, San Jose, CA, USA). All

samples were read in triplicates, with 10,000 events per read. For the IgM analysis, the population was divided according to Fluorescein isothiocyanate (FITC) signal intensity into low (10^0-$10^{0.5}$ FITC signal intensity), medium ($10^{0.5}$-10^1 FITC signal intensity) and high (10^1-10^4 FITC signal intensity). For PI analysis the population was divided into negative (10^0-10^1 fluorescence level (FL) units) and positive (10^1-10^4 FL units) populations. For the double stain analysis the population was divided according to FITC and PI fluorescence intensities into quadrants as described in Figure 2.1.

Figure 2.3 Quadrant division for IgM-PI double stained population analysis.



2.16 Staining slides preparation

Between 300 and 400 µl of a suspension containing $1x10^6$ cells/ml were applied to slides using a sterile cell funnel, with a piece of cardboard placed between the funnel and slide to absorb excess

fluids. The slides were then centrifuged using the Shandon Cytospin II cytocentrifuge (Thermo Fisher Scientific, Waltham, MA, USA) at 80 G for 5 minutes. The funnel and cardboard were removed carefully to avoid disturbing the cells, which formed a circle on the slide, roughly half a centimeter in diameter.

2.17 Wright-Giemsa staining

Wright-Giemsa stain was applied to the slide and incubated at room temperature for 4 minutes. The slide was then incubated with PBS for 10 minutes more. The slides was then rinsed with PBS and dried at room temperature. The stain was done by the Diagnostic Services in our institiute (Atlantic Veterinary College, University of Prince Edward Island, Charlottetown, PE, Canada)

2.18 Electron Microscopy

Cells from BoF were collected in 1.5 ml Eppendorf tubes, fixed in 2% glutaraldehyde, and treated with 1% OsO_4 in 0.1 M sodium cacodylate at 4°C for 1 hour. The samples were dehydrated with ethanol gradient washes from 50% to 100%, followed by propylene oxide treatment. They were then embedded in 100% Spurrs Resin and heated overnight at 65°C. Sections of 2 μm were placed on copper grids and examined at 80 kV with a transmission electron microscope.

2.19 Calculations and Statistical Analysis

Exact amplification efficiencies of target and reference genes were verified separately before normalizing the expression of the target gene to that of the housekeeping gene. Briefly, mean relative expressions of triplicates of each gene were calculated based on the expression of the housekeeping gene, β-actin, using Pfaffl's formula (Pfaffl, M.W. 2001). Gene expression after 4

or 8h incubation was presented as fold changes relative to the control. Gene expression following 1 or 10 µg/ml LPS treatment was presented as fold changes relative to the control group. Gene expression following 4 hour incubation in growth media was presented as fold changes relative to the 0h incubation group. Gene expression fold changes, standard error (SE), and statistical significance were calculated using REST 2009 (Qiagen, Toronto, ON, Canada) (Pfaffl, M.W. 2001). The relative expression ratios were used to determine differences in gene expression among different groups using a general linear model ANOVA test.

A 2x3 ANOVA design was used to analyze the effect of incubation time and FA on mRNA levels. The same design was used to analyze the effect of embryonic age and incubation time on cell viability, sIgM expression and double stained cells. A 3X3 ANOVA design was used to analyze the effect of LPS and FA on the mRNA levels. The ANOVA analysis was followed by a post hoc Tukey test if interactions between the main effects were significant. Test was corrected using the Bonferroni correction for multiple comparisons. Associations between factors were tested using the Pearson correlation method. All results were considered significantly different at $P < 0.05$. ANOVA, Tukey and Pearosn's correlation were all performed using Minitab 17 statistical software (Minitab Inc., State College, PA, USA).

2.20 Statistical designs

2.20.1 Association between FA and proximal promoter methylation of TLR2b, Igβ and MHCII β chain

$$Y_{x1} = \beta_0 + r\beta_{1x1} + \varepsilon_{x1\kappa}$$

Where: Y_{x1} = number of methylated cytosines that are part of a cytosine guanine dinucleotide (CpG) / total number of CpGs in the examined region X 100 (% Methylation); β_0 = % Methylation with no treatment; r = correlation coefficient; β_{1x1} = FA concentration in mM and $\varepsilon_{x1,x2}$ = experimental error.

2.20.2 Association between percent of promoter methylation and mRNA levels of TLR2b, Igβ and MHCII β chain

$$Y_{x1} = \beta_0 + r\beta_{1x1} + \varepsilon_{x1\kappa}$$

Where: Y_{x1} = mRNA levels of the gene normalized to reference gene expression (mRNA levels); β_0 = mRNA levels with no treatment; r = correlation coefficient; β_{1x1} = % Methylation and $\varepsilon_{x1,x2}$ = experimental error.

2.20.3 The Effect of incubation time and FA on mRNA levels of TLR2b, Igβ and MHCII β chain

$$Y_{x1x2} = \beta_0 + \beta_{1x1} + \beta_{2x2} + (\beta_1\beta_2)_{x1x2} + \varepsilon_{x1x2\kappa}$$

Where: $Y_{x1,x2}$ = response of mRNA levels of each gene normalized to housekeeping mRNA levels (mRNA level response) ; β_0 = mRNA levels when the main effects are 0; β_{1x1} = the effect of FA on mRNA levels; β_{2x2} = the effect of incubation time on mRNA levels; $(\beta_1\beta_2)_{x1x2}$ = the effect on mRNA levels due to interaction between FA concentration and incubation time and $\varepsilon_{x1,x2}$ = experimental error.

2.20.4 The effect of LPS and FA on the mRNA levels of TLR2b, Igβ and MHCII β chain

$Y_{x1x2}=\beta_0+\beta_{1x1}+\beta_{2x2}+(\beta_1\beta_2)_{x1x2}+\varepsilon_{x1x2\kappa}$

Where: $Y_{x1,x2}$ = mRNA level response; β_0 = mRNA levels when the main effects are 0; β_{1x1} = the effect of FA on mRNA levels; β_{2x2} = the effect of LPS on mRNA levels; $(\beta_1\beta_2)_{x1x2}$ = the effect on mRNA levels due to interaction between FA and LPS and $\varepsilon_{x1,x2}$ = experimental error.

2.20.5 The effect of FA on the mRNA levels of TLR2b, Igβ and MHCII β

$Y_{x1} = \beta_0+\beta_{1x1}+\varepsilon_{x1\kappa}$

Where Y_{x1} = mRNA level response; β_0 = mRNA levels with no treatment; β_{1x1} = mRNA level response to FA treatment and ε_{x1} = experimental error.

2.20.6 The effect of FA on mRNA levels after treatment with LPS

$Y_{x1} = \beta_0+\beta_{1x1}+\varepsilon_{x1\kappa}$

Where Y_{x1} = mRNA level response; β_0 = mRNA levels with no treatment; β_{1x1} = mRNA level response to FA treatment and ε_{x1} = experimental error.

2.20.7 The effect of ED and incubation time on the percent of live cells

$Y_{x1x2}=\beta_0+\beta_{1x1}+\beta_{2x2}+(\beta_1\beta_2)_{x1x2}+\varepsilon_{x1x2\kappa}$

Where: Y_{x1x2} = response in the proportion of live cell population; β_0 = response when the main effects are 0; β_{1x1} = the effect of ED on live cell population; β_{2x2} = the effect of incubation time on

live cell population; $(\beta_1\beta_2)_{x1x2}$ = the effect on percent of live cells due to interaction between ED and incubation time and $\varepsilon_{x1,x2}$ = experimental error.

2.20.8 The effect of ED and incubation time on the proportions of the cell populations expressing low, medium and high levels of surface IgM

$$Y_{x1x2}=\beta_0+\beta_{1x1}+\beta_{2x2}+(\beta_1\beta_2)_{x1x2}+\varepsilon_{x1x2\kappa}$$

Where: Y_{x1x2} = response in the proportion of cell populations expressing low, medium and high levels of surface IgM (sIgM expression); β_0 = response when the main effects are 0; β_{1x1} = the effect of ED on sIgM expression; β_{2x2} = the effect of incubation time on sIgM expression; $(\beta_1\beta_2)_{x1x2}$ = the effect on sIgM expression due to interaction between ED and incubation time and $\varepsilon_{x1,x2}$ = experimental error.

2.20.9 The effect of incubation time and FA on mRNA levels of the reduced folate carrier (RFC), TLR2b, Igβ and MHCII β mRNA levels

$$Y_{x1} = \beta_0+\beta_{1x1}+\varepsilon_{x1\kappa}$$

Where Y_{x1} = mRNA level response; β_0 = mRNA levels with no treatment; β_{1x1} = mRNA level response to incubation time and ε_{x1} = experimental error.

2.20.10 The effect of ED and incubation time on the proportions of live and dead cell populations expressing low, medium and high levels of surface IgM

$$Y_{x1x2}=\beta_0+\beta_{1x1}+\beta_{2x2}+(\beta_1\beta_2)_{x1x2}+\varepsilon_{x1x2\kappa}$$

Where: $Y_{x1,x2}$ = response in the proportion of live and dead cell populations expressing low, medium and high levels of surface IgM (double stained cell populations) ; β_0 = response when the main effects are 0; β_{1x1} = the effect of ED on double stained cell populations; β_{2x2} = the effect of incubation time on double stained cell populations; $(\beta_1\beta_2)_{x1x2}$ = the effect on double stained cell populations due to interaction between ED and incubation time and $\varepsilon_{x1,x2}$ = experimental error.

2.20.11 The effect of ED and FA on the percent of live cells population, proportions of sIgM expression and double stained cell populations

$$Y_{x1x2}=\beta_0+\beta_{1x1}+\beta_{2x2}+(\beta_1\beta_2)_{x1x2}+\varepsilon_{x1x2\kappa}$$

Where: $Y_{x1,x2}$ = response in the proportion of live cell population; β_0 = response when the main effects are 0; β_{1x1} = the effect of ED on live cell population; β_{2x2} = the effect of FA on live cell population; $(\beta_1\beta_2)_{x1x2}$ = the effect on mRNA levels due to interaction between ED and incubation time and $\varepsilon_{x1,x2}$ = experimental error.

Table 2.1 Primers used for cloning TLR2b, Igβ and RFC

Genes	Accession no.	Primer sequences (5' to 3')	Annealing temperature (°C)	Product size (bp)
TLR2b	NM_001161650.1	CATGCGATGCCACTCAGTCA	55	797
		CCACTTTCCAGTGCCCAAGA		
Igβ	NM_001006328.2	GACAGCAAGAACCTCACGGA	55	702
		GGTCACCAGCATAGCACCTT		
RFC	NM_001006513.1	ACCGGAAACATCTGGCTCTG	57	431
		CCATCCATCCGTCTGTCCTG		

TLR – Toll like receptor; Ig – Immunoglobulin; MHCII – Major Histocompatibility complex; RFC - Reduced folate carrier

Table 2.2 Primers used for real time PCR for TLR2b, Igβ, MHCII β chain and β actin mRNA gene expression levels

Genes	Accession no.	Primer sequences (5' to 3')	Starting annealing temp. (°C)	Product size (bp)
RFC	NM_001006513.1	TCTTCGGGGTCAACACATTCTT	58	152
		CATCAGCAGGTAGACCAGTGC		
TLR2b	JN544177	CGTTTGGCTCTCAGGGGAAA	55	388
		ATGGCAGAGTGCAGAAGGTC		
Igβ	NM_001006328.2	GGAAAGGCCTGAGGAAGACC	56	318
		GGATGCAACCAAGCAAGAGC		
MHCII β chain	NM_001044679.2	CCACGGACGTGATGCAGAAC	50	321
		CTGAGCGACTTCTTGGGGAG		
β actin	NM_205518.1	CAACACAGTGCTGTCTGGTGGTA	55	205
		ATCGTACTCCTGCTTGCTGATCC		

TLR – Toll like receptor; Ig – Immunoglobulin; MHCII – Major Histocompatibility complex; RFC - Reduced folate carrier

Table 2.3 Primers used for PCR post bisulfite conversion for the amplification of the proximal promoter region of TLR2b, Igβ, MHCII β chain genes.

Genes	Accession no.	Primer sequences (5' to 3')	Starting annealing temp. (°C)	Product size (bp)
TLR2b	NW_003763738.1	GGTAAAATGAATTAATTAATTTTTGAAA	53.5	312
		CCCATAAAAAAACAATTACATAAC		
Igβ	NW_003764296.1	GGTGTTTAAGTTTATGGAGTAAAGGTAGGT	63.5	661
		TACATAACTTTACAAACCCAAAATTAACATTACC		
MHCII β chain	NW_003763991.1	GAATATGGGGTTATTATGGTTATAT	56.5	324
		CCAACAATACCACCAACA		

TLR – Toll like receptor; Ig – Immunoglobulin; MHCII – Major Histocompatibility complex

3. In vitro epigenetic characterization of the effect of folic acid on the proximal promoter area and mRNA gene expression of B cell receptors in the chicken DT-40 cell line

3.1 Abstract

FA is one of the principal nutritional methyl group donors involved in the DNA methylation process, an epigenetic control mechanism generally associated with gene expression control. In the present study, the effect of FA on the proximal promoter methylation profile and expression of TLR2b, Igβ and MHCII β chain in chicken B cells was examined. DT-40 cell line cultures were incubated with 0, 1.72 or 3.96 mM of FA for 4 or 8 hours (h). The cells incubated for 8h were treated with 0, 1 or 10 µg/ml of LPS in triplicate. Positive association was found between FA concentration and percent of Igβ promoter methylation. A negative association between FA concentration and percent of MHCII β chain promoter methylation was demonstrated at 4h. Incubation time affected Igβ and MHCII β expression resulting in downregulation of the two genes after 8h compared to 4h. At 4h incubation, 1.72 mM FA upregulated TLR2b and 3.96 mM FA upregulated MHCII β chain expression. At 8h, 3.96 mM FA downregulated TLR2b and upregulated Igβ expression. A significantly higher TLR2b expression was observed with a combination of 1.72 mM FA for 4 hours. Under LPS treatment 1.72 mM FA–1 µg/ml LPS combination upregulated Igβ expression, while 3.96 mM FA-10 µg/ml LPS downregulated the same gene. Treatment with LPS had a significant downregulatory effect on TLR2b expression. Taken together, it is possible to infer that FA has a potential immunomodulatory effect on chicken B cells, possibly affecting their recognition abilities by both TLR and BCR pathways, as well as their ability to present antigen through the MHCII presentation pathway.

3.2 Introduction

In recent years, the importance of epigenetic mechanisms in regulation of immune system gene expression has been extensively studied (Baumann, K. 2015). These types of modifications, which include chromatin remodeling, histone tail modification, DNA methylation, non-coding RNA and microRNA gene regulation, acting alone or in combination, have the potential to effect gene expression in an organism, and are meiotically and mitotically heritable (Gyorffy, B. et al. 2016). One of the most widely studied mechanisms of epigenetic modifications is DNA methylation, the enzymatic addition of a methyl group to cytosines found in CpG dinucleotides (Ettig, R. et al. 2011). Methylating cytosines in the DNA sequence will, in most cases, protect from undesired gene expression by either recruiting histone methylation or deacetylation enzymes to alter the chromatin formation in the loci (Fuks, F. et al. 2000) or by directly interfering with binding of transcription factors (TF) (Tate, P.H. and Bird, A.P. 1993). The binding ability of some TFs, like specific protein (SP)1, are unaffected by the methylation status of their binding site (Holler, M. et al. 1988).

The methyl group originates from several nutritional cofactors such as betaine, choline, methionine or FA (Obeid, R. 2013). The latter, a natural dietary methyl donor, has been shown to improve health (Preynat, A. et al. 2010), performance (Preynat, A. et al. 2010), and embryonic development (Wang, X. et al. 2015).

The regulation of gene expression is particularly crucial in regard to the immune system, which is important in protecting the organism from any foreign insult, but if left unchecked, could cause harm to the host in the form of tissue damage (Hanash, A.M. et al. 2012) and autoimmune disease

(Salomon, B. et al. 2000). The genes participating in host defense are controlled by an array of stringent activating conditions, TFs (Szabo, S.J. et al. 2000), inhibitors (Stamper, C.C. et al. 2001) and activators (Stetson, D.B. and Medzhitov, R. 2006). In recent years, studies have shed light on the role of epigenetic mechanisms in general (Liu, Y. et al. 2015), and DNA methylation in particular (Pacis, A. et al. 2015) in maintaining immune system regulation and homeostasis.

One of the principal participants in host defense in the chicken is the B cell population, which express cell surface receptors that are associated with both the innate and adaptive immune systems. In the context of the innate response, B cells express TLRs, a family of pattern recognition receptors that recognize and bind specific MAMPS (St Paul, M. et al. 2012a). With regard to the adaptive immune system, the BCR complex includes an antigen capture moiety (sIg) and a signal transfer moiety. The latter is made up of two non-covalently bound proteins named Igα and Igβ, which are involved in the activation of the cell and the rigorous selection process of B cells in the BoF (Pike, K.A. and Ratcliffe, M.J. 2005). Furthermore, B cells constitutively express MHCII (Muhlethaler-Mottet, A. et al. 1997), which allows processing of captured antigens that culminates in presentation of peptides in the context of the MHCII complex – a heterodimer made up of α and β chains (Baecher-Allan, C. et al. 2006). T-independent antigens, such as LPS, do not have to be processed and presented to T cells to elicit antibody production. This MAMP is recognized by the TLRs on B cells without involving the BCR or the MHCII system, resulting in the production of low affinity antibodies (Pone, E.J. et al. 2015). Furthermore, recognition of MAMPs by TLRs can be followed by a cross linking and engagement between the TLRs and BCR that can lead to antibody class switching in both T cell independent (Macpherson, A.J. et al. 2000) and dependent manners (Matter, M.S. and Ochsenbein, A.F. 2008).

The importance of the B cell's role in both the innate and adaptive immune system through their receptors and effectors mechanism, as well as the known influence of FA on immunological pathways, support additional research into the effect of FA on the capacity of B cells to recognize, process and present antigen. Therefore, the current study was conducted to assess the effect of FA on the proximal promoter methylation profile and gene expression of TLR2b, BCR Igβ and MHCII β chain in chicken DT-40 B cell line.

3.3 Materials and Methods

The materials and methods used to examine the effect of FA and LPS challenge on DT-40 B cell line culture are described in chapter 2. Briefly, cells were incubated for either 4 or 8 h with 0, 1.72 or 3.96 mM of FA. After 4 h 0, 1 or 10 µg/ml LPS was added to the cultures in the 8 h incubation group for the duration of the experiment. The expression of TLR2b, Igβ and MHCII β chain was examined and compared to the expression of the house keeping gene β-actin. The effect of FA on the methylation status of the promoter region of the genes of interest was examined using DNA bisulfite conversion followed by PCR and sequencing.

3.4 Results

3.4.1 Proximal promoter region methylation patterns of TLR2b, Igβ and MHCII β chain

The effects of FA incubation on the methylation patterns of the proximal promoter regions of TLR2b, Igβ and MHCII β chain are presented in Fig. 1. The single CpG dinucleotide in the promoter region of the TLR2b gene positioned at -160 from the start codon remained methylated when the cells were exposed to 0, 1.72 or 3.96 mM of FA, for either 4 or 8h of incubation (Fig.

3.1 A). The promoter region of the Igβ gene (Fig. 3.1 B) contains 13 CpG dinucleotides. Positions -193, -180, -138, -135, -73 and -52 remained unmethylated and positions -104, -67 and -60 remained methylated regardless of FA concentration and incubation time. Position -101 was unmethylated only when the cells were incubated without FA for 4h. Position -82 was methylated only when the cells were incubated with 3.96 mM of FA for 4h. Lastly, position -50 was methylated only when the cells were incubated with either 1.72 or 3.96 mM of FA for 8h. The MHCII β chain gene promoter region (Fig. 3.1 C) contains 24 CpG dinucleotides. The cytosines situated at -173, -131, -125, -108, -94, -91, -88, -85 and -82 remained unmethylated while positions -55, -49, -40, -30, -18, -14, and -11 remained methylated regardless of FA concentration and incubation time. At 4h incubation, position -166 remained methylated while it was unmethylated after 8h regardless of FA concentration. Cytosines at the -158 and -101 positions were methylated when incubated with either no FA or 1.72 mM of FA for 4h, and were unmethylated under all other conditions. Positions -98 and -62 were unmethylated only when the cells were exposed to 3.96 mM FA. The cytosine positioned at -61 was methylated except when incubated for 4h without FA or with 3.96 mM FA. Lastly, CpG dinucleotides situated at -47 and -38 bases upstream of the start codon were methylated except when incubated without FA for 8h.

Figure 3.1. Proximal Promoter area methylation of TLR2b, Igβ and MHCII β chain.

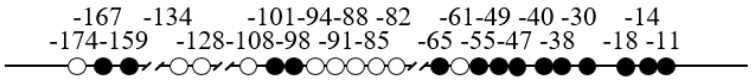
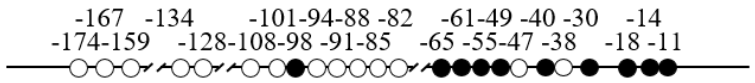
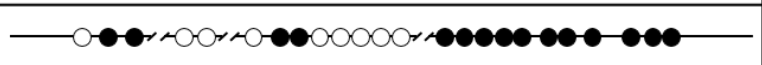
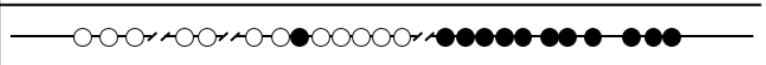
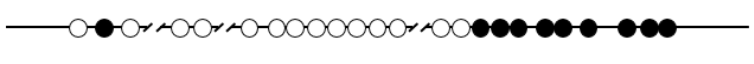
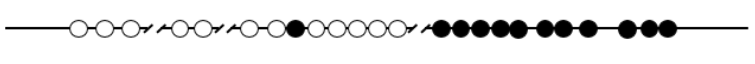
A

Folic acid concentration (mM)	Incubation time (hours) 4	8
0	-160	-160
1.72	-160	-160
3.96	-160	-160

B

Folic acid concentration (mM)	Incubation time (hours) 4	8
0	-193 -180 -138 -135 -104 -101 -97 -82 -73 -67 -60 -52 -50	-193 -180 -138 -135 -104 -101 -97 -82 -73 -67 -60 -52 -50
1.72		
3.96		

C

FA concentration (mM)	Incubation time (hours)	
	4	8
0		
1.72		
3.96		

(A) TLR2b, (B) Igβ, and (C) and MHC β chain. Each circle represents a single CpG dinucleotide. Methylated and unmethylated cytosines are shown by (●) and (○), respectively. Numerical values represent the location of the cytosine relative to the start codon. A break in the line indicates a sequence of at least 15 base pairs without CpG dinucleotides.

3.4.2 Association between FA concentration and percent of proximal promoter methylation of TLR2b, Igβ and MHCII β chain

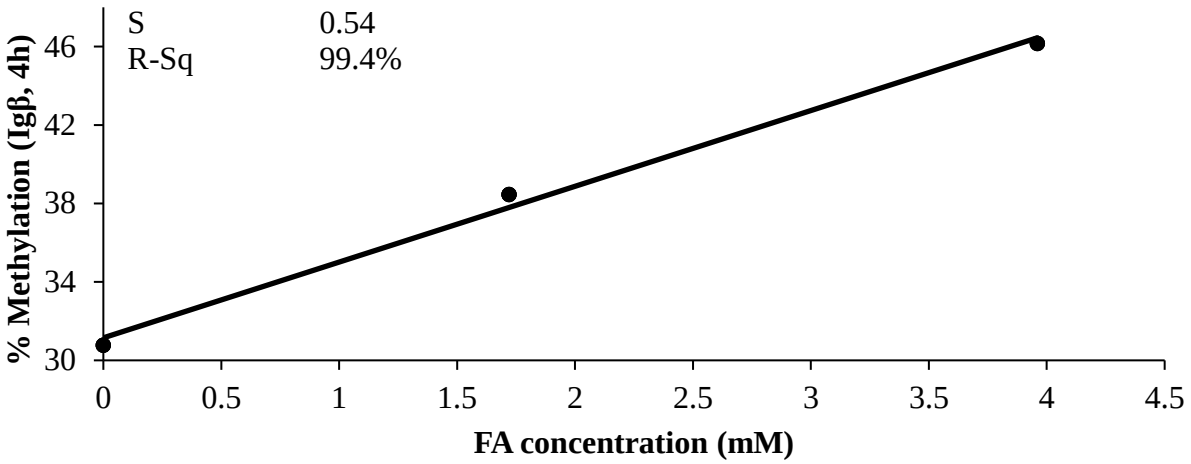
Only promoter regions with changes in methylation are presented. A positive association was found between FA concentration and percent of methylation of the Igβ promoter region at both 4h incubation (Fig. 3.2 A, $r=0.997$, $P < 0.05$) and 8h incubation (Fig. 3.2 B, $r=0.83$, $P < 0.05$). However, a negative association was found between FA concentration and percent of MHCII β chain promoter methylation after 4h incubation (Fig. 3.2 C, $r=-0.666$, $P < 0.05$).

3.4.3 Effect of incubation time and FA concentration on TLR2b, Igβ and MHCII β chain mRNA levels

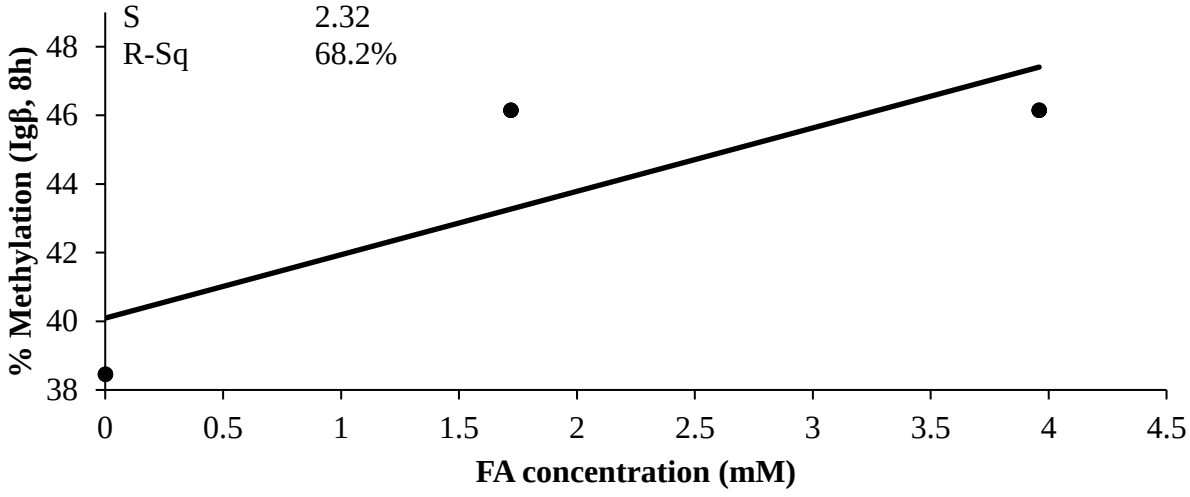
The effect of incubation time and FA concentration on mRNA expression of TLR2b, Igβ and MHCII β chain are presented in Table 3.1. While the incubation time had a significant downregulatory effect on the mRNA levels of Igβ and MHCII β chain ($P < 0.05$), it did not have a significant effect on the mRNA levels of TLR2b ($P > 0.05$). An interaction effect of incubation time by FA concentration on TLR2b mRNA level was observed. A Post hoc Tukey test (Fig. 3.3) revealed that in the 1.72 mM FA treatment, TLR2b mRNA level was higher than the level in the control group after 4h incubation than after 8h incubation ($P < 0.05$). No significant interaction effect of FA concentration by time was observed on the expression of the other genes of interest. FA concentration had a significant effect on TLR2b mRNA levels ($P < 0.05$), with expression at 1.72 mM FA significantly higher than that observed at 3.96 mM FA. FA concentration did not have a significant effect on the mRNA levels of the other genes of interest ($P > 0.05$).

Figure 3.2. FA concentration association with percent of Igβ and MHCII β chain promoter methylation.

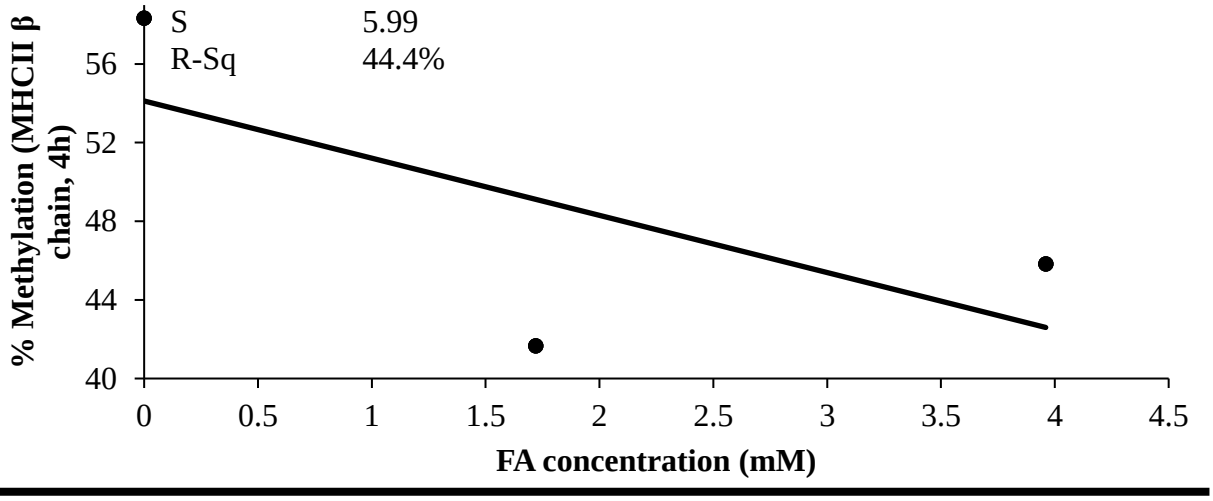
A



B



C



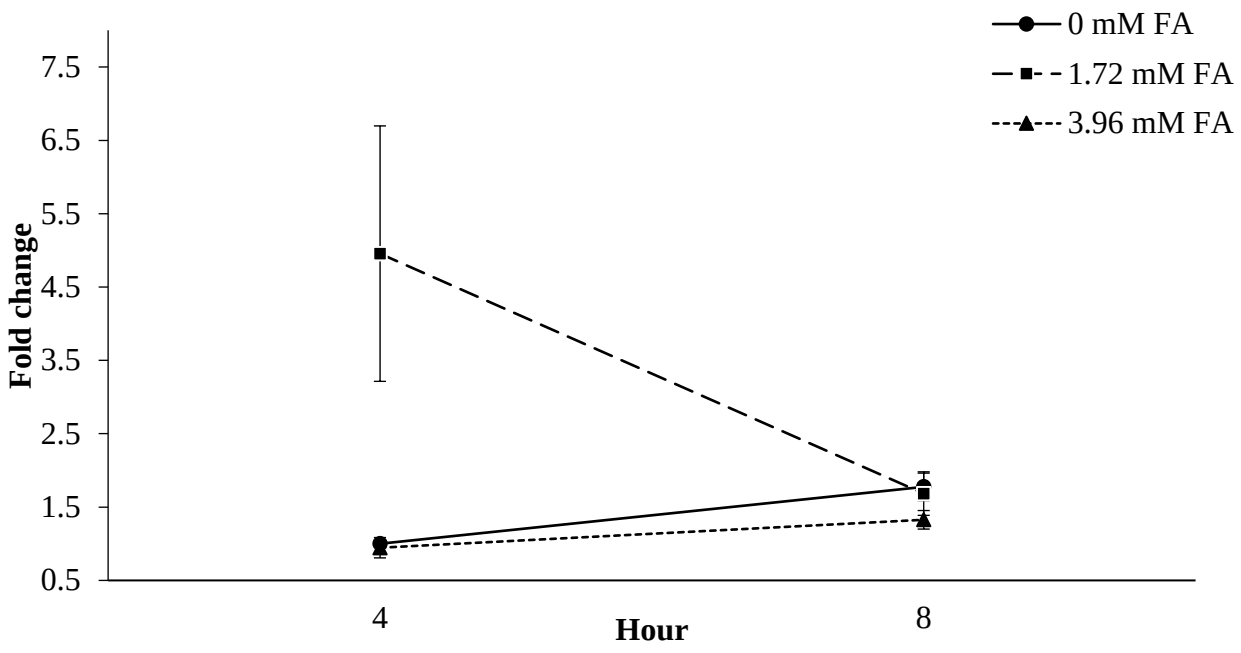
Significant association plots between FA concentration and percent of promoter methylation for (A) Igβ after 4 hours (r^2=0.994) (B) Igβ after 8 hours (r^2=0.682) and (C) MHCII β chain after 4 hours (r^2=0.444).

Table 3.1 FA conc. and incubation time effect on TLR2b, Igβ and MHCII β chain expression

	Time (h)		FA (mM)			SEM	P values		
Gene	4	8	0	1.72	3.96		Time	FA	Time × FA
TLR2	2.3	1.6	$1.39^{(ab)}$	$3.32^{(a)}$	$1.14^{(b)}$	0.42	0.259	0.022	0.031
Igβ	$1.02^{(a)}$	$0.36^{(b)}$	0.65	0.6	0.81	0.09	<0.001	0.141	0.684
MHCII β chain	$1.89^{(a)}$	$0.33^{(b)}$	0.7	1.36	1.28	0.26	<0.001	0.243	0.141

Values represent mean relative gene expression from three independent experiments repeated three times, normalized to the expression of the reference gene (β actin). Means sharing no common superscript letter differ significantly ($P < 0.05$).

Figure 3.3. Interaction plot for FA concentration and incubation time effect on TLR2b expression.



Effect of incubation time and FA concentration combinations on TLR2b expression. Points represent mean relative expression levels from three independent experiments repeated three times. Lines represent standard error (SE) Differences are significant at ($P < 0.05$).

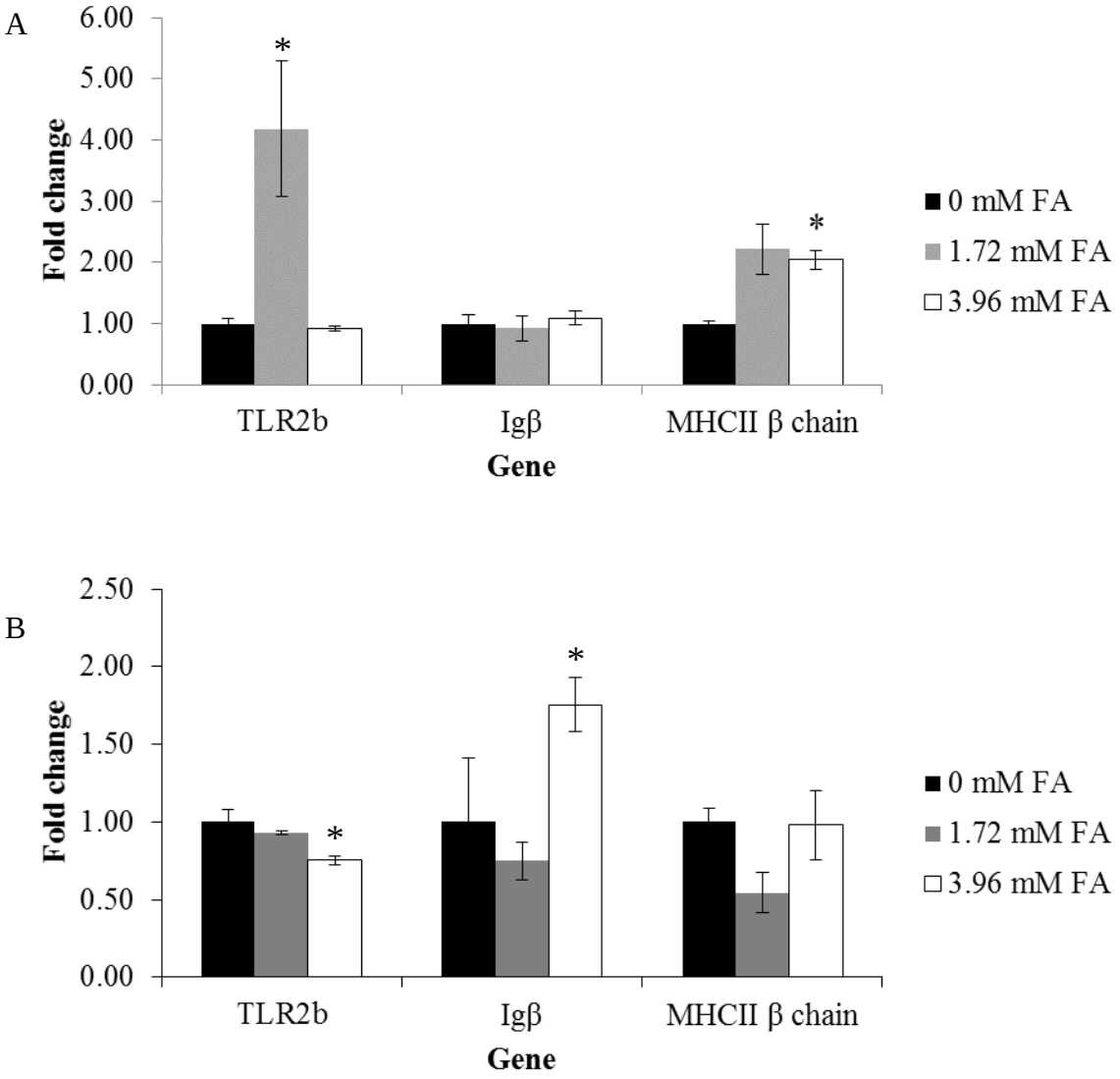
3.4.4 The effect of FA concentration at 4 and 8 h incubation time on mRNA levels of TLR2b, Igβ and MHCII β chain

The effect of each FA concentration on mRNA levels after 4h or 8h of incubation time are presented in Fig. 3.4. Compared to control, when incubated for 4 hours, 1.72 mM FA significantly upregulated the expression of TLR2b, while 3.96 mM FA significantly upregulated the mRNA level of the MHCII β chain gene (Fig. 3.4 A. $P < 0.05$ and $P < 0.05$, respectively). After 8h incubation, 3.96 mM FA significantly downregulated the mRNA level of TLR2b while upregulating that of Igβ (Fig. 3.4 B. $P < 0.05$ and $P < 0.05$ respectively). No significant changes were observed for the other genes of interest ($P > 0.05$).

3.4.5 The effect of LPS on expression of TLR2b, Igβ and MHCII β chain

The effect of LPS and FA concentration on mRNA levels TLR2b, Igβ and MHCII β chain is presented in Table 3.2. An interaction effect of FA by LPS concentrations was observed on Igβ expression of ($P < 0.05$). The post hoc Tukey test revealed that the highest expression of Igβ was achieved after a 1.72 mM FA and 1 μg/ml LPS treatment combination (Fig. 3.5) although no combination was significantly different than the others ($P > 0.05$). There were no significant interactions of FA by LPS concentrations observed on TLR2b and MHCII β chain. LPS treatment resulted in significant TLR2b downregulation ($P < 0.05$) while no change in Igβ and MHCII β chain was observed ($P > 0.05$).

Figure 3.4. Expression of TLR2b, Igβ and MHCII β chain following incubation with FA.



Values presented as relative fold change after normalization to reference gene (β actin) following a (A) 4h or (B) 8h incubation period. Values shown are means of triplicates of three independent experiments. Lines represent SE. * indicate significant difference from control group (0 mM FA for 4 or 8h, respectively) ($P<0.05$).

3.4.6 The effect of FA concentration and LPS treatment on expression of BCR

The effect of each FA concentration on mRNA levels of the genes of interest under both 1 and 10 µg/ml LPS treatments are presented in Fig. 3.6. Treatment with 1.72 mM of FA and treatment with 1 µg/ml of LPS resulted in a significant upregulation of Igβ mRNA levels compared to the control ($P < 0.05$) while no significant change was observed in TLR2b, and MHCII β chain expression ($P > 0.05$) (Fig. 3.6 A). Similarly, there was no significant ($P>0.05$) change in gene expression in the group treated with 10 µg/ml LPS (irrespective of FA concentration), except for a downregulation of Igβ with 3.96 mM FA treatment ($P < 0.05$) (Fig. 3.6 B).

3.4.7 Association between percent of proximal promoter methylation and gene expression

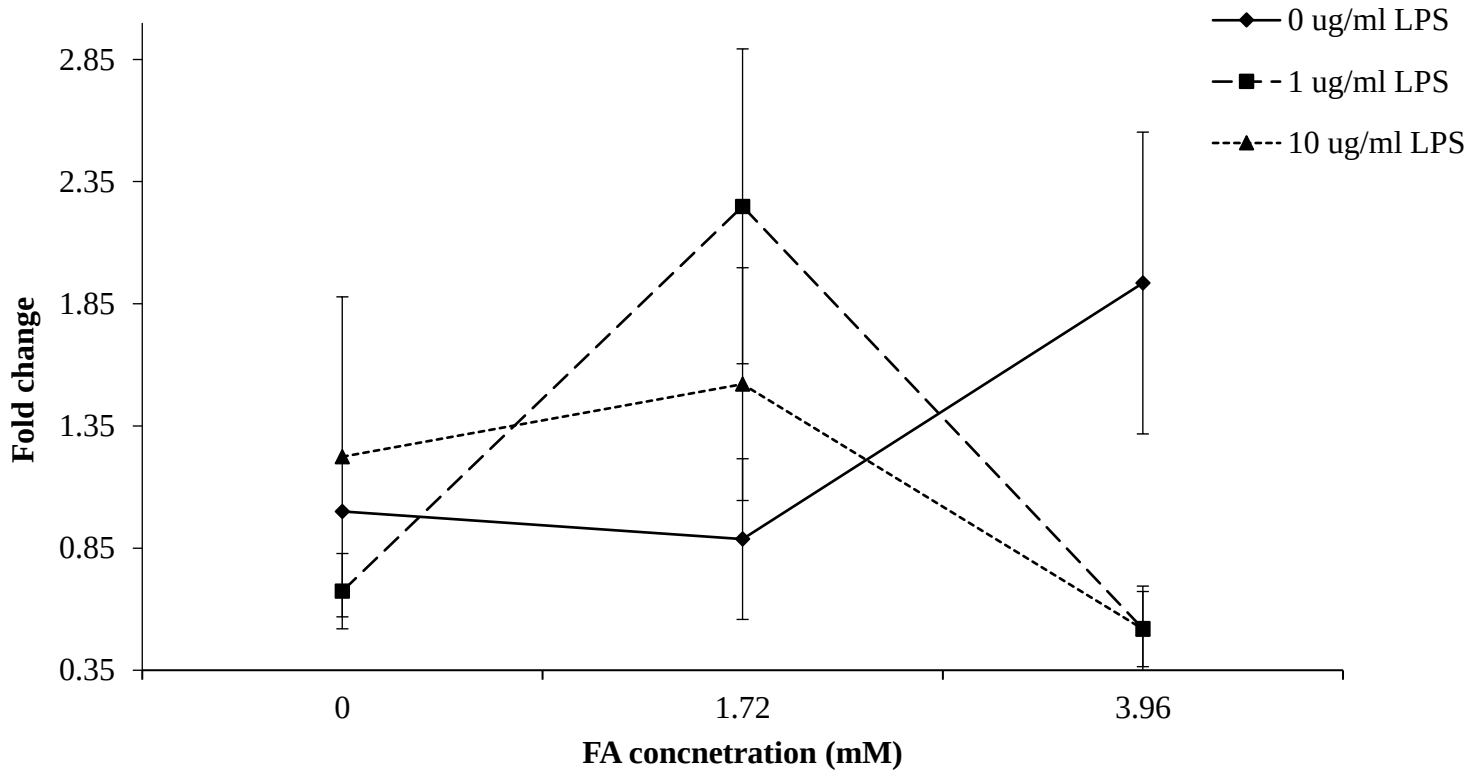
No significant association was observed between the methylation percent of the promoter region of the genes of interest and their respective mRNA levels, regardless of incubation time. Association between the methylation percent of the promoter region and TLR2b expression was not tested as there was no change in the methylation profile in the promoter area of that gene.

Table 3.2 Effect of FA and LPS conc. on TLR2b, Igβ and MHCII β chain expression

	LPS (µg/ml)			FA (mM)			SEM	P values		
Gene	0	1	10	0	1.72	3.96		LPS	FA	LPS × FA
TLR2	0.9[(a)]	0.62[(b)]	0.58[(b)]	0.7	0.71	0.65	0.03	<0.001	0.33	0.404
Igβ	1.27	1.14	1.08	1	1.55	0.99	0.16	0.863	0.19	0.036
MHCII β chain	0.87	1.12	1.27	1.3	0.98	1.03	0.08	0.134	0.33	0.247

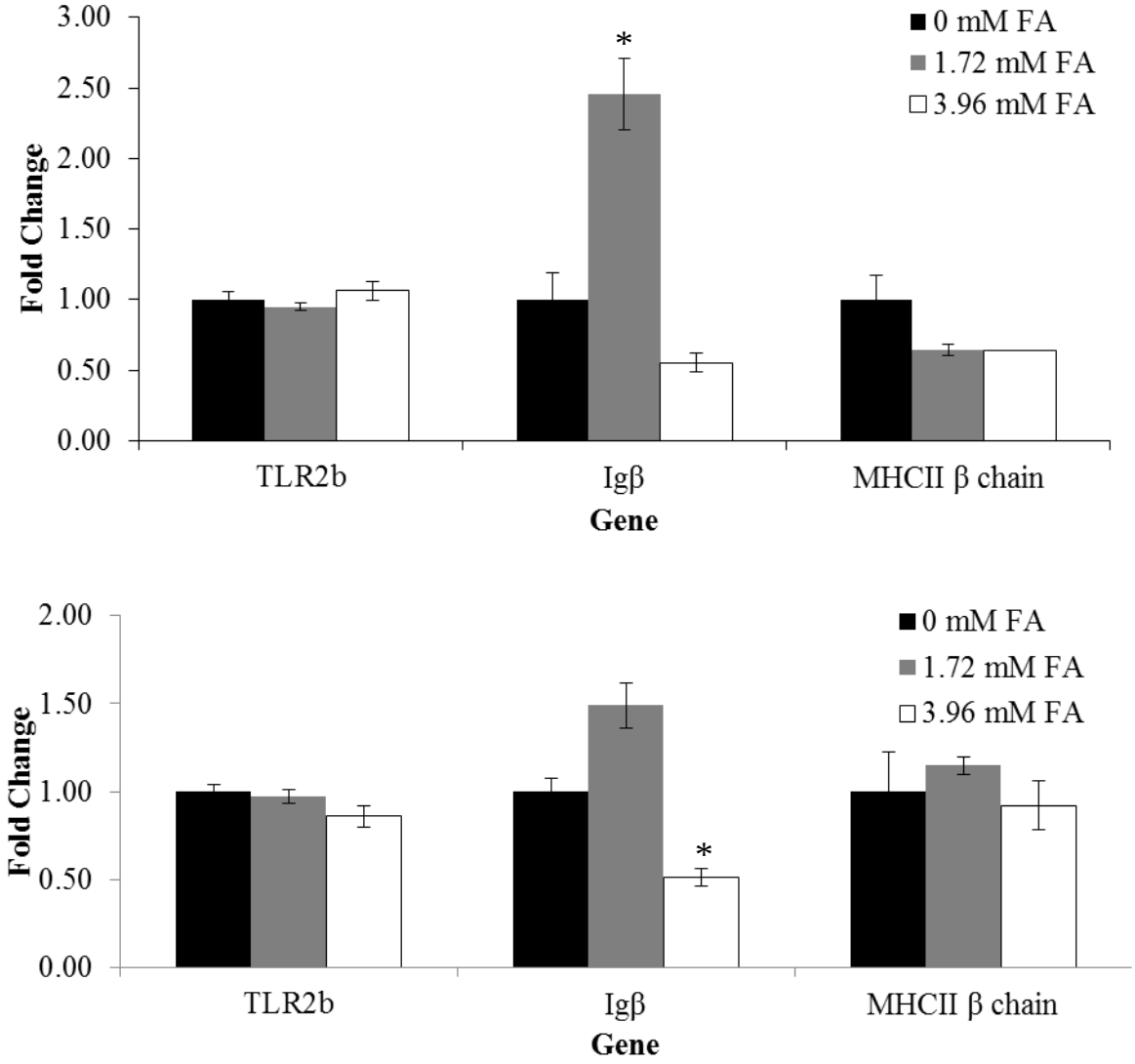
Values represent mean relative gene expression from three independent experiments repeated three times, normalized to the expression of the reference gene (β actin). Means sharing no common superscript letter differ significantly ($P < 0.05$).

Figure 3.5. Interaction plot for FA and LPS conc. effect on Igβ expression.



Effect of FA and LPS concentration combinations on Igβ expression. Points represent mean relative expression levels from three independent experiments repeated three times. Lines represent standard error. Differences are significant at ($P < 0.05$).

Figure 3.6. Expression of TLR2b, Igβ and MHCII β chain following treatment with FA and LPS.



Values presented as relative fold change after normalization to reference gene (β actin) after a 4h incubation with 0, 1.72 or 3.96 mM FA followed by 4h of (A) 1 µg/ml LPS or (B) 10 µg/ml LPS treatment. Values shown are means of triplicates of three independent experiments. Bars represent standard error. * indicate significant difference from control group (No FA with 1 µg/ml or 10 µg/ml, respectively) ($P<0.05$).

3.5 Discussion

TLR2b is one of the two avian orthologues to mammalian TLR2 (Temperley, N.D. et al. 2008). After dimerization with TLR1 or TLR6, the complex binds to several microbial components such as peptidoglycan, lipomannan and lipopeptides (Farhat, K. et al. 2008), all components of Gram-positive bacterial cell wall (St Paul, M. et al. 2012a). TLR2 also has the ability to recognize fungal (Farhat, K. et al. 2008), protozoan (Wong-Baeza, I. et al. 2010) and viral (Klein Klouwenberg, P. et al. 2009) ligands. In this experiment, methylation of the proximal promoter region of TLR2b was unaffected by FA, regardless of concentration or incubation time. The fact that there is only a single CpG dinucleotide in the examined region and that the methylation pattern of this CpG was unchanged throughout the experiment suggests that the expression of the TLR2b gene is not under direct proximal promoter methylation control. In fact, the same lack of relationship between the methylation pattern of the TLR2 promoter and the expression of the gene can be seen in human endothelial cells (Diesel, B. et al. 2012). Other instances where FA addition did not change the methylation pattern of the promoter region of a gene are documented (Kotsopoulos, J. et al. 2008). Although incubation with FA did not change the methylation profile of the proximal promoter region of the TLR2b gene in the current study, it upregulated its expression after 4h incubation with 1.72 mM FA. Instances where FA affected gene expression without changing the DNA methylation profile of their promoters have been demonstrated (Price, R.J. et al. 2015). Other possibilities are that the methylation pattern of different TLR2b regulatory region or regions, or even that of the gene itself, was affected by the treatment, which, in turn, affected the gene expression. This kind of upregulation can be beneficial for the immune system. Previous

publications have shown that injecting lipoteichoic acid (LTA) (a TLR2 ligand) *in ovo* caused an increase in pro-infalmmatory mediators and reduced infectious laryngotracheitis virus infection post hatch (Thapa, S. et al. 2015). Furthermore, trerating chicks intranasaly with a TLR2 ligand reduced avian influenze shedding post infection (Barjesteh, N. et al. 2015a). In the current study, incubation with 3.96 mM FA for 8h downregulated TLR2b expression. It is possible that the high concentration and/or incubation time resulted in the methylation of a different region, causing the described effect. LPS caused a downregulation of TLR2b. This type of downregulatory effect has been reported before, where a 2h LPS challenge downregulated TLR2 in the chicken liver (Zhang, Y. et al. 2013).

While the membrane bound immunoglobulins recognize antigen, Igβ, together with Igα, make up the signal transduction moiety of the complex (Bruhl, H. et al. 2015), and as such, are crucial for both B cell selection (Pike, K.A. and Ratcliffe, M.J. 2005) and activation (Van Kooten, C. et al. 1997). The results of this research show that the percentage of methylation in the proximal promoter region of the Igβ gene was positively associated with the amount of FA used, regardless of incubation time. A positive association between FA and percent of methylation was described before (Anderson, O.S. et al. 2012). In the current study, incubation with 3.96 mM FA for 8h significantly upregulated Igβ expression. This expression profile may fit a state favorable for antigen recognition by the BCR complex, Interestingly, in this study, a 1.72 mM FA and 1 µg/ml LPS combination resulted in upregulation of the Igβ gene, while a 3.96 mM FA and 10 µg/ml LPS combination resulted in its downregulation. It is possible to infer that a 3.96 mM FA and 10 µg/ml LPS combination shifts the receptor expression in B cells in a way that favors antigen binding by

the TLR pathway, rather than the BCR pathway. It has been reported previously that binding of T cell independent antigens, such as Macrophage activating lipopeptides (MALP-2) (Borsutzky, S. et al. 2005) and *Staphylococcus aureus* Protein A (Bekeredjian-Ding, I. et al. 2007) (both of them TLR2 ligands), to TLR receptors on B cells may result in the production of low affinity IgM antibodies, without antigen presentation to T cells (Chen, X. et al. 1997). These "natural" antibodies play a major role in the primary line of defense against infections (Casali, P. and Schettino, E.W. 1996), and findings from the current study may point to the beneficial role of FA in readying the B cells for the production of this type of antibodies.

It has been speculated previously that other than the pathway leading to natural antibody production, a TLR-BCR crosslink can induce B cell activation and production of antigen specific antibodies (Pone, E.J. et al. 2012b). Whichever of these pathways are activated, the production of antibodies is independent of antigen presentation to effector T cells positive for CD4 protein expression (Matter, M.S. and Ochsenbein, A.F. 2008), and depends on the nature of the antigen (Ochsenbein, A.F. and Zinkernagel, R.M. 2000). On the other hand, recognition through a BCR cross link, or a joint TLR-BCR engagement may also result in a T dependent B cell activation (Jendholm, J. et al. 2009). After the T cell is activated by recognizing the antigen-MHCII complex through the T cell receptor (TCR), it will produce and secrete cytokines (Damdinsuren, B. et al. 2010). These cytokines will induce affinity maturation processes of the antibodies produced by the B cell, as well as proliferation and differentiation of the cell (Palm, N.W. and Medzhitov, R. 2009). For this reason, it was our aim to observe changes in the B cells ability to present antigens. The MHCII β chain gene is part of the class II antigen family that enables B cells to present antigens

to effector CD4+ T cells (Giles, J.R. et al. 2015). In the present study, a negative association between the FA concentration and the percent of methylation of the promoter region of the MHCII β chain was observed. Moreover, there was no association between MHCII β chain expression and the percent of promoter methylation. It has been demonstrated that genes belonging to the MHCII family in mammals present all the possible variations of association (positive, negative or non-existent) between CpG methylation and gene expression (Yuan, X.J. et al. 1994). An upregulation of MHCII β chain expression was observed when the B cells were incubated with 3.96 mM of FA for 4 hours, and incubation time with FA was a factor affecting MHCII β chain expression.

While it is possible that the effects observed in this experiment are due to a direct influence of FA on the promoter of MHCII β chain, it is also possible that FA incubation affected another MHCII regulatory gene like the class II, MHC trans-activator (CIITA) (Holling, T.M. et al. 2004). Therefore, more research is needed to establish the pathway in which FA affects the expression of the MHCII β chain gene. Since the effect of FA addition on methylation, and the effect of promoter methylation on gene expression are tissue, gene and sex specific (Anderson, O.S. et al. 2012), different FA supplementation regimens might be optimal for different poultry production systems and age of birds.

3.6 Conclusions

In conclusion, incubation with FA is associated with changes in the methylation profile of the proximal promoter regions of Igβ and MHCII β chain, and modified expression of TLR2b, Igβ and MHCII β chain. However, FA concentration and exposure time seem to be integral factors in its effect. For cells not treated with LPS, incubation with FA might change the chicken B cells ability

to recognize antigen through the TLR2b pathway or the BCR. Furthermore, the inferred increase in MHCII expression may be indicative of improved antigen capture and presentation abilities by B cells. Incubation with high concentrations of FA in this experiment upregulated the MHCII β chain gene and Igβ gene while downregulating the TLR2b gene. This change in expression may fit the profile of B cells primed to present antigens to activated effector $CD4^+$ T cells. This interaction may initiate a series of activities that belong to the adaptive immune response, which includes B cell proliferation and differentiation, and affinity maturation of the antibodies produced by the cell. On the other hand, this interaction will initiate cytokine production by the effector T cell, which will regulate the immune response. The results presented here support previous findings published by our group regarding the role of FA as a dietary immunomodulator (Munyaka, P.M. et al. 2012), and strengthens the notion that immune modifications can be achieved through epigenetic mechanisms.

4. *Ex vivo* epigenetic characterization of the effect of folic acid on the proximal promoter area and mRNA gene expression of chicken B cell receptors in chicken cells harvested from the bursa of Fabricius

4.1 Abstract

FA is a vitamin that acts as a principal nutritional methyl group donor involved in the DNA methylation process, an epigenetic mechanism associated with gene expression control. The aim of this study is to examine the effect FA addition has on the promoter methylation profile and expression TLR2b, BCR Igβ and MHCII β chain in embryonic BoF chicken B cells, as well as whether it has an effect on cell viability and surface IgM levels. Cultured BoF cells have been known to lose viability rapidly. Doubling the amount of chicken serum in the growth media helped maintain the cells viability for the duration of the experiment. At ED15, removing the cells from the BoF environment for 4 hours lowered the population of $IgM^{low/med}$ cells and increased the population of IgM^{high} cells, accompanied by an upregulation of the Igβ gene expression. At ED18 TLR2b expression was downregulated, and at ED21 TLR2b and MHCII β chain expression were downregulated, as well as the expression of the reduced folate carrier (RFC), a major FA transporter. To examine the effect of FA, cells taken from ED15, 18 and 21 were incubated with either 0, 1.72 or 3.96 mM FA for 4 hours. The result indicate that FA did not have an effect on cell viability. Treatment with 3.96 mM FA at ED15 reduced the population of IgM^{med} cells, and both concentrations did the same at ED18. At ED21, 3.96 mM FA increased the IgM^{high} population. At ED15, both concentrations caused a downregulation of Igβ, and at ED18 1.72 mM FA caused a downregulation of RFC expression. The promoter methylation profile was not affected by FA or incubation time. The results demonstrate the immune modulating capabilities of FA in the developing B cell, the importance of the bursal environment in B cell development and a novel method for maintaining the viability of embryonic chicken B cells in an ex vivo culture.

4.2 Introduction

Infectious diseases have a significant impact on the poultry industry due to mortality, decreased production and risk of contamination leading to zoonotic diseases (Patterson, J.A. and Burkholder, K.M. 2003). Until recently, the solution was prophylactic treatment with antibiotics added to the feed (Leitner, G. et al. 2001). However, in recent years the use of these growth promoters has come under scrutiny due to concerns regarding antibiotic resistance (Witte, W. 2000), the effect on nutrient absorption (Knarreborg, A. et al. 2004) and the effect on normal gut microbiota (Yitbarek, A. et al. 2013). As a result of these concerns the use of antibiotic growth promoters has been banned in several countries (Capita, R. and Alonso-Calleja, C. 2013). This became a driving force to find alternative solutions that can be used to help protect the animal from the wide variety of pathogens.

One solution is nutritional intervention. Past research has shown the link between specific nutrients such as vitamins (Katona, P. and Katona-Apte, J. 2008) and amino acids (Kidd, M.T. et al. 2001) and the immune capabilities of the chicken. FA is an example of a nutrient that has been shown to have an effect on the chicken immune capabilities, including T cell populations (Wintergerst, E.S. et al. 2007) and cytokine expression (Munyaka, P.M. et al. 2012). It is recognized by two types of transporters. The first is the proton coupled folate carrier (PCFT), which mediates FA absorption in the upper part of the intestine and works best under low pH conditions (Visentin, M. et al. 2014). The second type of transport is the RFC, which is more diversely expressed (Whetstine, J.R. et al. 2002), has a higher affinity for its ligand than the PCFT (Zhao, R. et al. 2001) and its activity is optimal under neutral pH conditions (Sierra, E.E.

et al. 1997). While this compound is used by the organism for DNA synthesis (Crider, K.S. et al. 2012), it is also a methyl donor, which is utilized in the process of DNA methylation. This enzymatic process involves adding a methyl group to the 5' carbon of cytosines that are part of a cytosine-guanine dinucleotide in the DNA (Ghoshal, K. and Bai, S. 2007). This process can happen anywhere on the DNA including in the promoter region of a gene. When cytosines are methylated in this region they act as both steric interference for the binding of transcription factors, as well as a recruitment site for proteins that further inhibit the availability of the gene for transcription (Russ, B.E. et al. 2013). This process is part of a family of mechanisms protecting the organism from unwanted gene expression and are named epigenetic mechanisms, as they control gene expression though the alteration of the structure of the genetic material, as opposed to changes to its sequence (Chuang, J.C. and Jones, P.A. 2007).

While many cells participate in the protection of the organism, the chicken B cell has a unique role in the immune reaction. It expresses receptors that allow the cell to function under the mantle of both the innate and adaptive immune systems. B cells express TLRs, a family of pattern recognition receptors that recognize and bind MAMPs (St Paul, M. et al. 2012a). Such a recognition will result in the rapid production of low affinity antibodies by the B cell, independent of interactions with T cells (Pone, E.J. et al. 2012a). B cells also express BCR, which is made up of an antigen capture moiety, the sIg, and a signal transfer moiety (Chu, P.G. and Arber, D.A. 2001). Igα and Igβ are non-covalently bound proteins that make up the signal transduction portion that is involved in the activation of the cell once the antigen is recognized (Lee, W.Y. and Tolar, P. 2013). The BCR and TLRs can interact. This interaction will result in class switching of the antibodies produced by the B cell, also without any need for interaction

with T cells (Pone, E.J. et al. 2010). Conversely, if an antigen is recognized by cross linked BCRs, it will be internalized, processed and presented in the context of the MHCII, once that complex is recognized by CD4 positive helper T cells, it will cause the B cells to go through antibody class switching and somatic hypermutation, which will improve the affinity of the produced antibody towards the presented antigen. The interaction between the cells will also signal the B cells to differentiate into plasma cells and memory cells (Pone, E.J. et al. 2012b).

While many cell types like T cells (Chen, C.H. et al. 1994) and macrophages (Garceau, V. et al. 2015) follow the same developmental route in chickens as the one known in mammals, the B cells follow a different developmental path, using the BoF instead of the bone marrow (Ciriaco, E. et al. 2003). Situated between the sacrum and the cloaca (Casteleyn, C. et al. 2010), this sac-like formation is visible from ED5. During development, folds are formed, which are colonized between ED10 and ED15 by B cell precursors (Nagy, N. et al. 2004). These cells will continue to develop and proliferate, and by the time of hatch (day 21), 90% of cells in this organ will be IgM positive (McCormack, W.T. et al. 1991). These cells go through a two stage selection process to ensure that they can be activated by the BCR complex (van der Burg, M. et al. 2002), and that they are not highly responsive against self-antigens (Luning Prak, E.T. et al. 2011). For reasons not entirely clear, roughly 95% percent of these cells go through apoptosis (Paramithiotis, E. et al. 1995). Around hatch, mature naïve B cells emigrate from the BoF to the periphery where they can fulfil their role as immune cells.

One of the TLRs expressed by the chicken B cell is TLR2b. While other TLRs work as homodimers to recognize a specific MAMP, TLR2b binds to either TLR1 or TLR6 to recognize

a wide variety of molecular patterns, including those components of Gram-positive bacteria, Gram-negative bacteria, fungi (Farhat, K. et al. 2008), protozoa (Wong-Baeza, I. et al. 2010) and viruses (Klein Klouwenberg, P. et al. 2009). In chickens, TLR2 mediated recognition activates anti-viral responses (Barjesteh, N. et al. 2015a) and both Th1 and Th2 like responses (St Paul, M. et al. 2012b). This versatility in recognition as well as downstream activation makes TLR2 ligands a powerful adjuvant. This can be seen in the immune responses in chickens to avian influenza (Barjesteh, N. et al. 2015a) and laryngotracheitis vaccines (Thapa, S. et al. 2015), in both mature animals and *in ovo* (Thapa, S. et al. 2015).

The need for alternatives to antibiotic growth promoters, the importance of the B cell in protecting the organism through the use and interactions of three types of receptors (TLRs, BCR and MHCII) combined with the presence of an organ solely dedicated to the development and selection of these cells warrant the examination of the effect of FA on the methylation status of the promoter regions of these genes, and the effect it has on their expression during the B cell maturation process.

4.3 Materials and Methods

The materials and methods used to examine the effect of FA on B cells harvested from embryonic BoF are described in chapter 2. Briefly, cells were harvested from BoF at embryonic ages 15 (30 BoF), 18 (10 BoF) and 21 (4 BoF). Cells were either immediately analyzed (0 hour) or incubated for 4 hours with 0 mM, 1.72 mM or 3.96 mM FA, with $1x10^6$ cells per treatment. The cells were stained for surface IgM, propidium iodide (PI) or both, and analyzed using flow cytometry. The expression of TLR2b, Igβ and MHCII β chain was compared to the expression of the house keeping

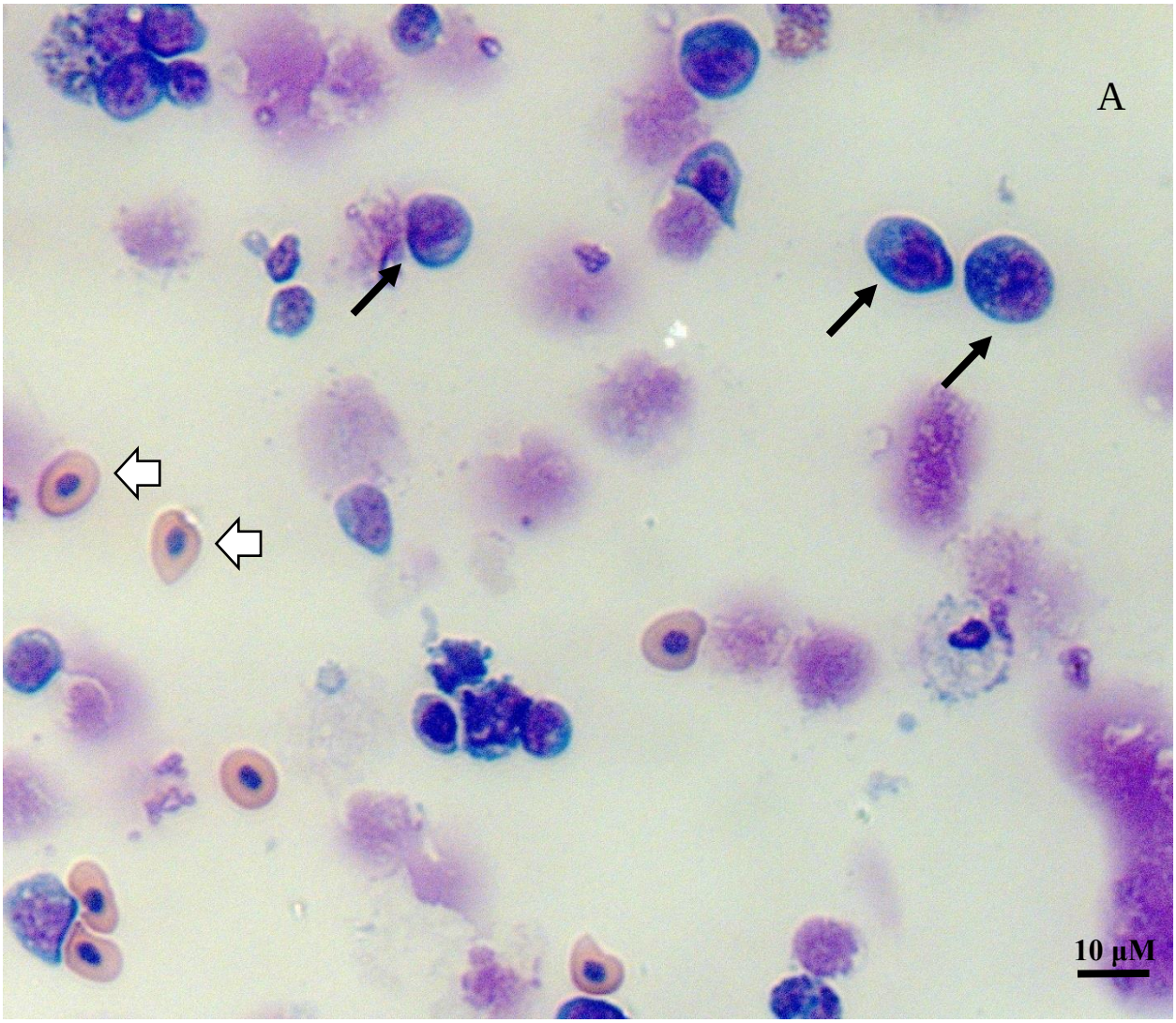
gene β-actin. The effect of FA on the methylation statues of the promoter region of the genes of interest was examined using DNA bisulfite conversion.

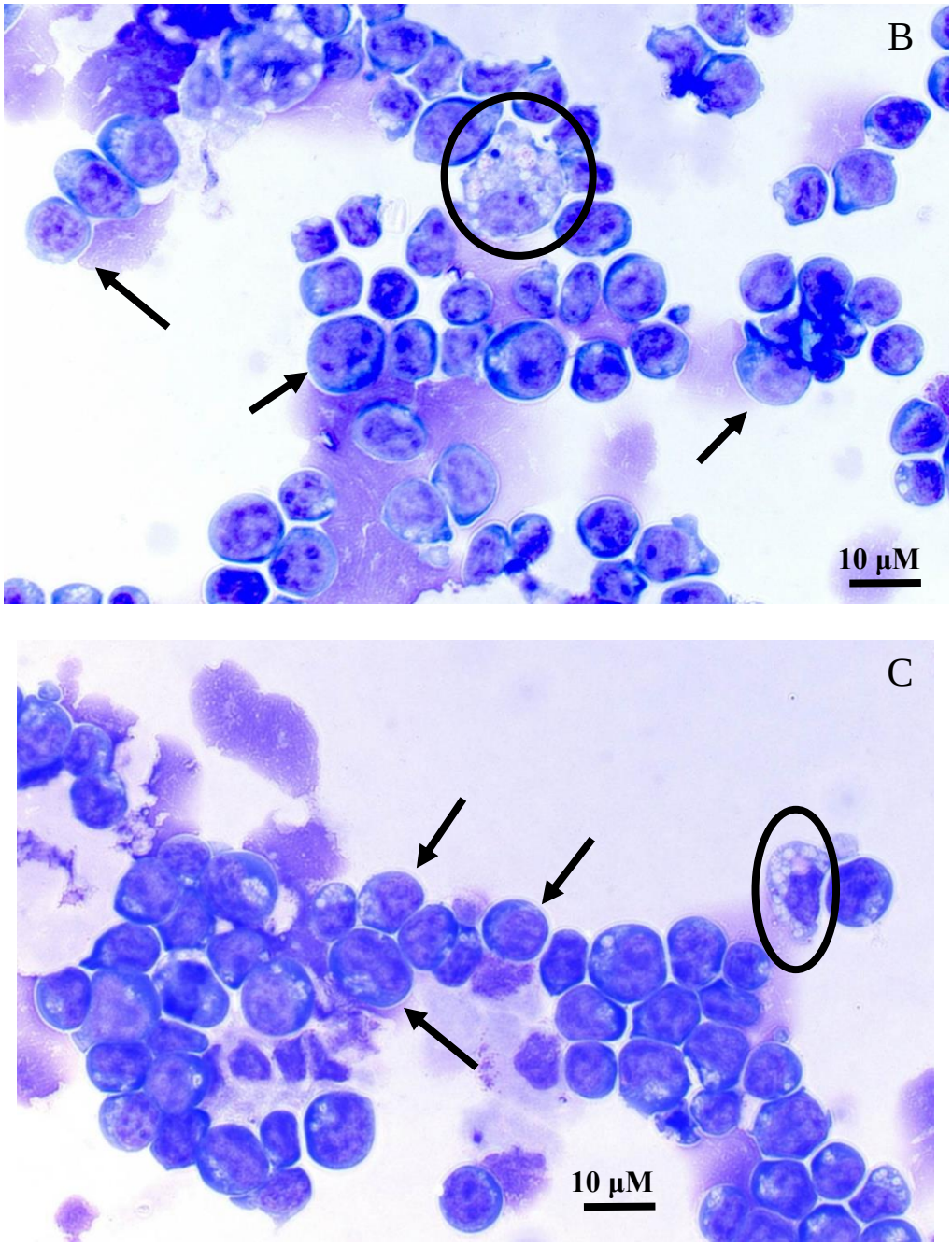
4.4 Results

4.4.1 Wright-Giemsa stain for cells extracted from BoF at embryonic day 15, 18 and 21

In all images, cells can be seen with a high nucleus to cytoplasm ratio, marked in black arrows. A few red blood cells can be seen in the image of ED15, marked with white arrows (Fig. 4.1 A). Cells with a high nucleus to cytoplasm ratio are also seen in the images taken from ED18 and ED21 (Fig. 4.1 B and C, respectively). A macrophage is visible in the ED18 and ED21 images, circled in black.

Figure 4.1 Wright-Giemsa stain of cells extracted from embryonic BoF at different ages





Cells from each time point were stained with Wright-Giemsa and observed with light microscopy under 100X oil immersion lens. (A) ED15, (B) ED18, (C) ED21. Black arrows - cells with lymphocyte phenotype White arrows - Erythrocytes. Magnification, x

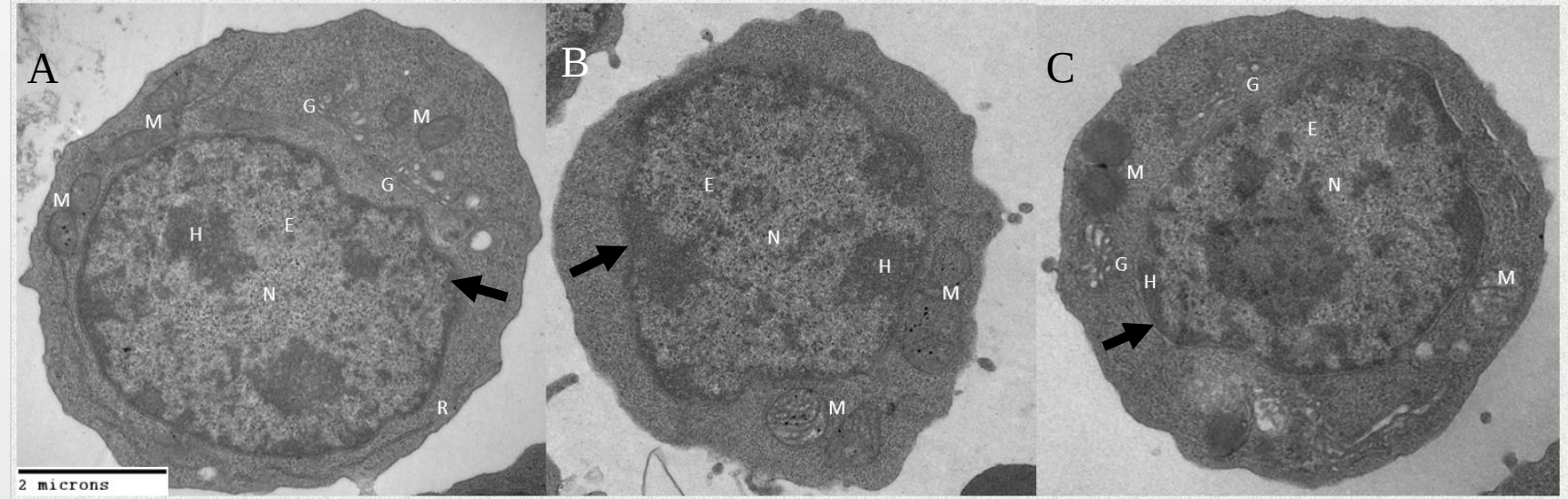
4.4.2 Transmission electron microscopy imaging of BoF cells at different embryo ages

Images of single cells taken after extraction from the BoF are presented in Figure 4.2. The three cells taken from ED15 (Fig. 4.2 A), ED18 (Fig. 4.2 B) and ED21 (Fig. 4.2 C) exhibit a high nucleus/cytoplasm ratio, although this is more noticeable in the image of the cell taken from an ED18 embryo. Rough endoplasmic reticulum is almost not apparent in the cells. Golgi complexes are visible in the cytoplasm of ED15 and ED21 cells. Lysosomes are visible in the cytoplasm of ED15 and ED18. The ED15 cell exhibits the most mitochondria than the other two cell types.

4.4.3 Effect of incubation time on embryonic chicken B cell viability

The effect of incubation time on cell viability for each embryonic age is presented in Table 4.1. A significant interaction between age and time was observed for the live cell population (Fig. 4.3) ($P < 0.05$). An ED21-4 hour combination had the highest percent of live cells than any other combination except for the ED21-0 hour combination. Age had a significant effect, as the percentage of live cells was significantly lower at ED15 than ED18 and ED21 ($P < 0.05$). The population of dead cells in the ED21-4 hour combination was significantly lower than all other combinations ($P < 0.05$). Age had a significant effect on this cell population as well, as the percentage of dead cells at ED15 was higher than that found in ED18 regardless of the incubation time. There was no significant difference between the populations of dead cells at ED18 and ED21 in the 0 h incubation group. However, after 4 hours the population of dead cells at ED18 was significantly higher than the one at ED21 ($P < 0.05$).

Figure 4.2 Transmission electron microscopy images of cells extracted from embryonic BoF at different ages



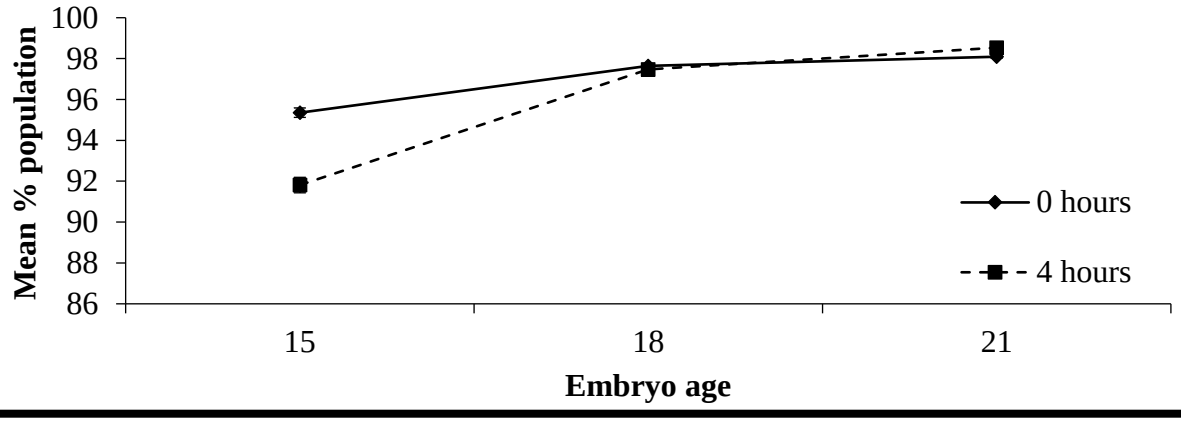
Transmission electron microscopy of cells extracted from BoF at (A) ED15, (B) ED18 and (C) ED21, X20, 000 magnification. Nuclear envelope is pointed at with black arrows. N - Nucleus H - Heterochromatin E - Euchromatin G - Golgi complex; M - Mitochondria; R - rough endoplasmic reticulum;

Table 4.1: Effect of incubation time on cell viability according to embryonic age

Status	Embryo age (days)			Time (hours)		SEM	P values		
	15	18	21	0	4		ED	Time	EDxT
Live	93.65$^{(a)}$	97.55$^{(b)}$	98.31$^{(c)}$	97.06$^{(a)}$	96.11$^{(b)}$	0.07	< 0.01	< 0.01	< 0.01

Values represent percent of live cell population means from three independent experiments repeated three times. Cells were examined with no incubation (0 hours) and after 4 hours incubation at ED15, 18 and 21. Means sharing no common superscript letter differ significantly (P < 0.05).

Figure 4.3 Interaction plot for the effect of incubation time and embryo age on cell viability



Points represent mean relative expression from three independent experiments repeated three times. The graphs represent interactions between embryo ages and incubation time effect on live embryonic B cell population. Lines represent SE. Significant differences (P < 0.05) between means were tested post hoc by Tukey's test.

4.4.4 Effect of incubation time on embryonic chicken B cell receptor-IgM (BCR)

The effects of incubation time on cell surface IgM expression by embryonic age are presented in Table 4.2. An interaction between age and incubation time was observed for all cell populations ($P < 0.05$). ED21-4 hour and ED18-0 hour combinations had a significantly higher population of IgM^{low} cells than any other combination (Fig. 4.4 A). Age had a significant effect on this population as it was significantly lower at ED15 than in the two other ages examined ($P < 0.05$). IgM^{med} cell population was significantly higher at ED21-0 hour than any other combination (Fig. 4.4 B). All other combination but ED15-4 hours are not significantly different from one another ($P > 0.05$). ED15-4 hours had the lowest level of IgM^{med} cells than any other combination ($P < 0.05$) (Fig. 4.4 B). The highest IgM^{high} cell population (Fig. 4.4 C) was expressed at ED15 after 4 h incubation ($P < 0.05$) followed by ED15-0 hour combination. ED21-0 hour and ED18-0 hour, while not significantly different from each other, had the lowest proportion of IgM^{high} cells ($P < 0.05$). Age had a significant effect on this population, being significantly higher at ED15 than the two other ages observed ($P < 0.05$).

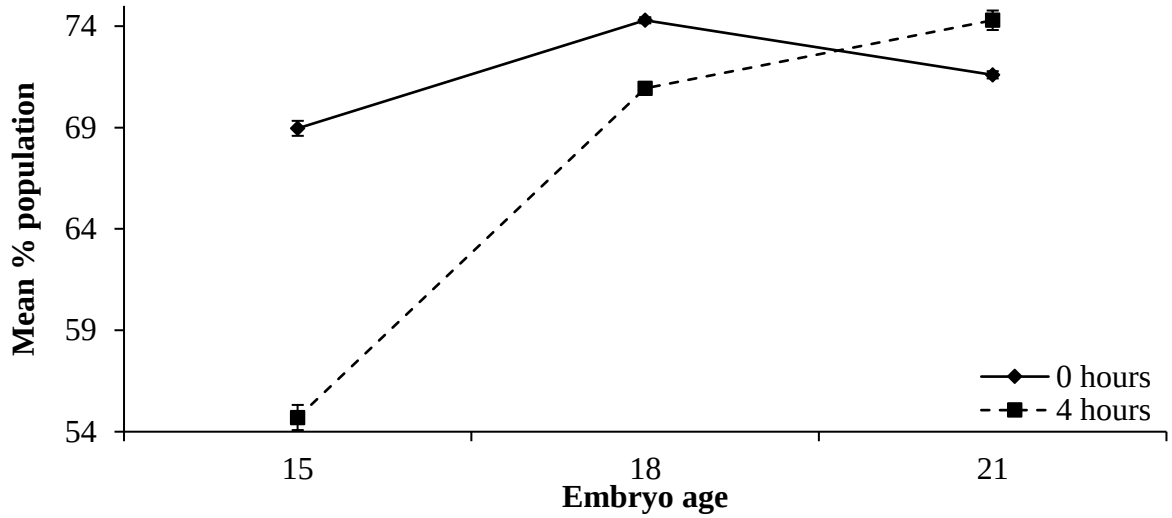
Table 4.2: Effect of incubation time on embryonic chicken BCR according to embryonic age

Population	Embryo age (days)			Time (hours)		SEM	P values		
	15	18	21	0	4		ED	Time	EDxT
IgM^{low}	$63.4^{(a)}$	$72.69^{(b)}$	$72.99^{(b)}$	$71.75^{(a)}$	$68.58^{(b)}$	0.11	< 0.001	< 0.001	< 0.001
IgM^{med}	$21.23^{(a)}$	$23.2^{(b)}$	$23.75^{(b)}$	$23.63^{(a)}$	$21.82^{(b)}$	0.09	< 0.001	< 0.001	< 0.001
IgM^{high}	$14.83^{(a)}$	$4.04^{(b)}$	$3.26^{(c)}$	$4.16^{(a)}$	$8.09^{(b)}$	0.08	< 0.001	< 0.001	< 0.001

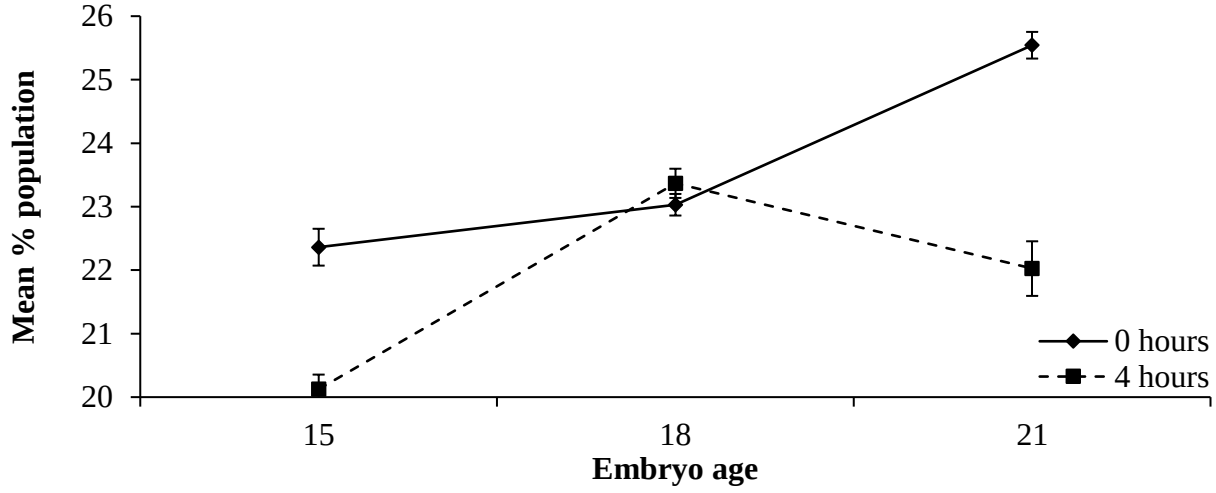
Values represent means of percent of cell population fitting each category from three independent experiments repeated three times. Cells were examined with no incubation (0 hours) and after 4 hours incubation at ED15, 18 and 21. Means sharing no common superscript letter differ significantly ($P < 0.05$).

Figure 4.4 Interaction plot for the effect of incubation time and embryo age on BCR expressing cell populations

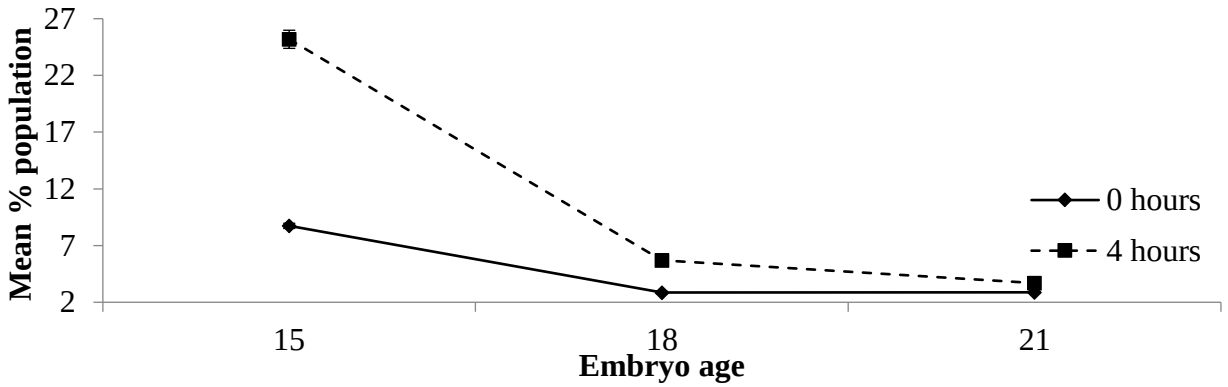
A



B



C



Points represent mean from three independent experiments repeated three times. The graphs represent the effect of age and time interactions on (A) IgM^{low}, (B) IgM^{med} and (C) IgM^{high} cell populations. Lines represent SE. Significant differences between age and time combinations were tested post hoc by Tukey's test. Differences between combinations in each cell population are significant at ($P < 0.05$).

4.4.5 Effect of incubation time on staining embryonic B cell populations for both surface IgM and Propidium Iodide

The effect of the age of the embryo, as well as the effect of incubation time on cell staining for both surface IgM and PI are presented in Table 4.3. An interaction was observed between age and time post extraction for all populations except dead $IgM^{low/med}$ cells. For the population of dead IgM^{high} cells, ED21-4 hours had a significantly lower percentage than both other ages examined ($P < 0.05$) (Fig. 4.5 A). However, 21-0 and 21-4 combinations are not significantly different from one another. The effect of age is significant, as the percent of dead IgM^{high} cells is higher at ED15 than ED18 and ED21 ($P < 0.05$). The population of live $IgM^{low/med}$ cells is highest at ED21 regardless of time post extraction and at ED18-0 hour combination (Fig. 4.5 B). ED15 has a significantly lower live $IgM^{low/med}$ cell population than that observed at the two other ages examined ($P < 0.05$). The live IgM^{high} cell population was highest at ED15 regardless of time post extraction (Fig. 4.5 C). However, ED18 and ED21 cells that were in growth media for 4 hours had a significantly higher population of Live IgM^{high} cells than ED18 and ED21 immediately post extraction. The population of dead $IgM^{low/med}$ cells was effected by age and time post extraction, with this population significantly declining as development progresses, and when incubated in growth media for 4 hours when compared to 0 hours (immediately post extraction) ($P < 0.05$).

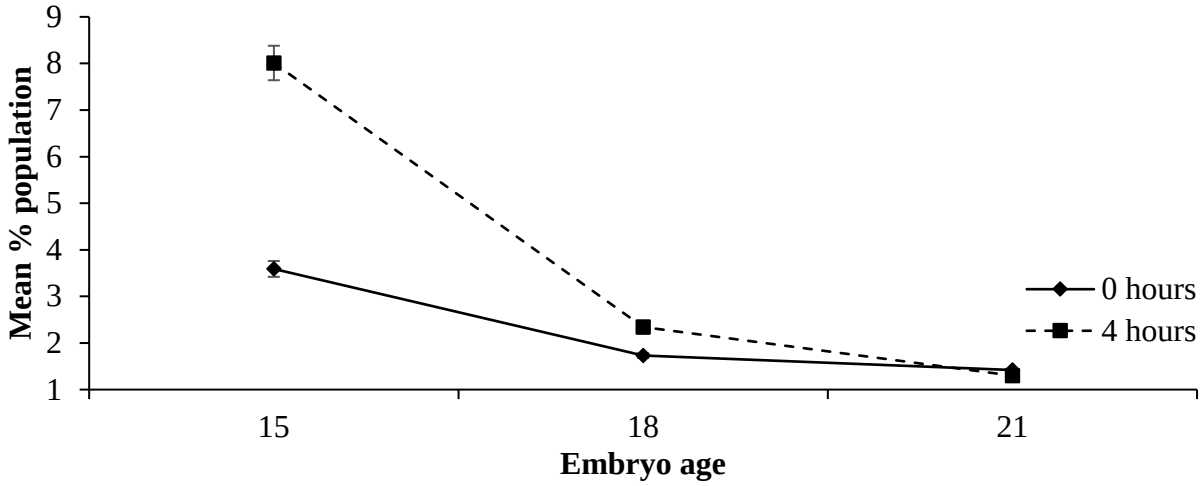
Table 4.3: Effect of incubation time on staining embryonic B cell populations for both surface IgM and PI according to embryonic age

Population	Embryo age (days)			Time (hours)			P values		
	15	18	21	0	4	SEM	ED	Time	EDxT
Dead $IgM^{low/med}$	$0.87^{(a)}$	$0.47^{(b)}$	$0.37^{(b)}$	$0.81^{(a)}$	$0.35^{(b)}$	0.02	< 0.001	< 0.001	0.269
Dead IgM^{high}	$5.36^{(a)}$	$2.01^{(b)}$	$1.36^{(c)}$	$2.06^{(a)}$	$2.9^{(b)}$	0.04	< 0.001	< 0.001	< 0.001
Live $IgM^{low/med}$	$83.64^{(a)}$	$95.25^{(b)}$	$96.34^{(c)}$	$94.55^{(a)}$	$90.08^{(b)}$	0.1	< 0.001	< 0.001	< 0.001
Live IgM^{high}	$9.32^{(a)}$	$1.95^{(b)}$	$1.85^{(b)}$	$2.02^{(a)}$	$5.17^{(b)}$	0.06	< 0.001	< 0.001	< 0.001

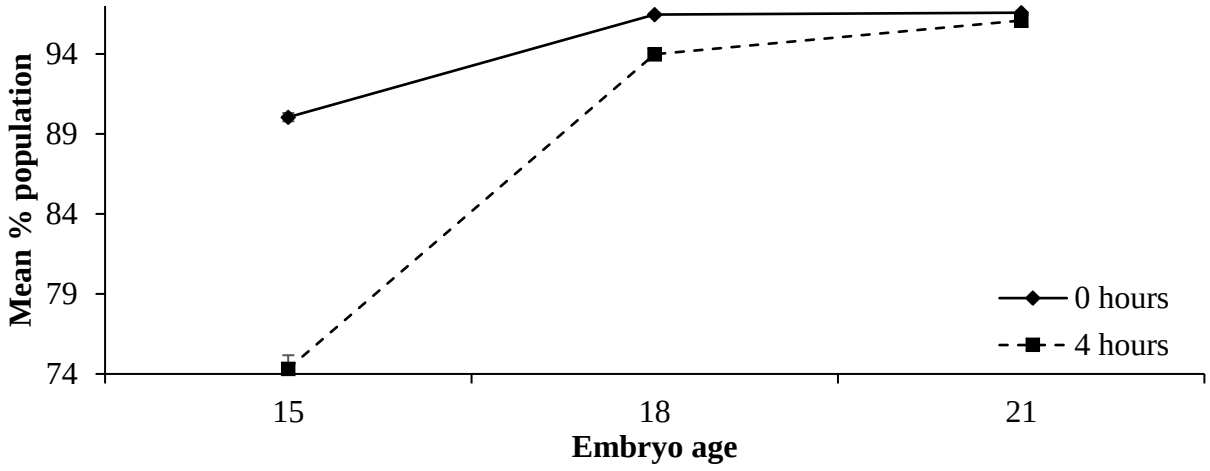
Values represent means of percent of cells found in each quadrant from three independent experiments. Cells were examined with no incubation (0 hours) and after 4 hours incubation at ED15, 18 and 21. Means sharing no common superscript letter differ significantly. Significant differences ($P < 0.05$) between means were tested post hoc by Tukey's test.

Figure 4.5 Interaction plot for the effect of incubation time and embryo age on staining embryonic B cell populations for both surface IgM and PI

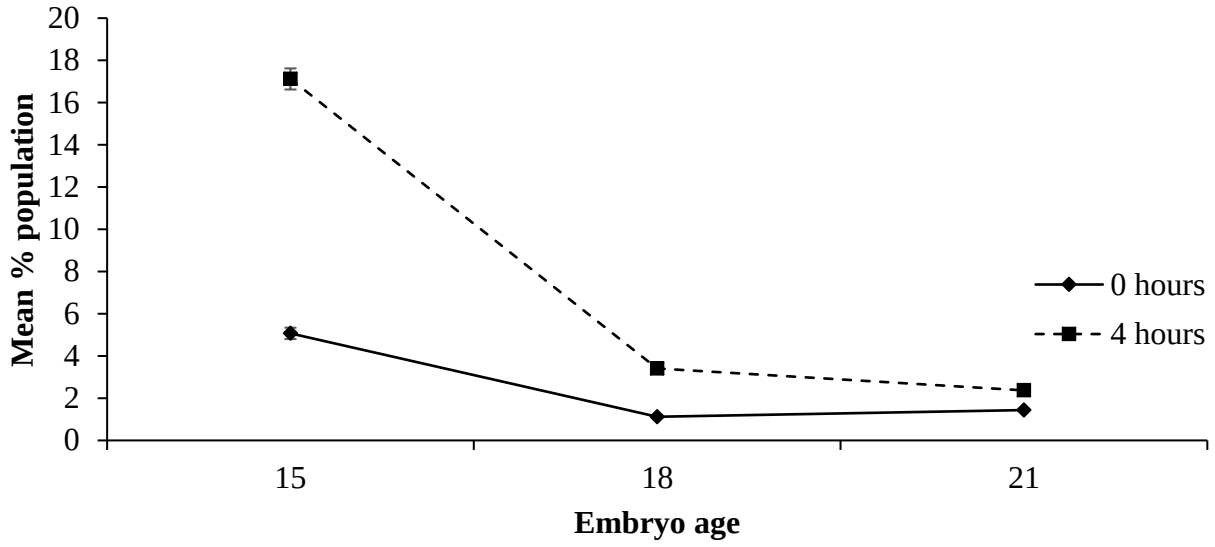
A



B



C



Points represent means from three independent experiments repeated three times. The graphs represent the effect of age and time interactions on (A) dead IgM^{high}, (B) live $IgM^{low/med}$ and (C) live IgM^{high} cell populations. Lines represent SE. Significant differences between age and time combinations were tested post hoc by Tukey's test. Differences between combinations in each cell population are significant at ($P < 0.05$).

4.4.6 Effect of FA on embryonic chicken B cell viability

The effects of FA on cell viability at different embryo ages are presented in Table 4.4. The age of the embryo had a significant effect on cell viability ($P < 0.05$), with the live cell population increasing as embryonic development progresses ($P < 0.05$). FA did not have a significant effect on cell viability ($P > 0.05$).

4.4.7 Effect of FA on embryonic chicken B cell receptor-IgM (BCR)

The effects of FA on BCR expression according to age are presented in Table 4.5. There was a significant interaction between FA and age for all cell populations ($P < 0.05$). The population of IgM^{low} cells was not significantly different at ED21 embryos regardless of FA concentration used. The IgM^{low} population at ED21 was not significantly different that that found in an ED18-3.96 mM FA combination ($P < 0.05$) (Fig. 4.6 A). The effects of embryo age on the IgM^{low} population can be observed for this population, as the IgM^{low} population was significantly lower at ED15 compared to the other two ages examined. Interaction was also observed for the IgM^{med} cell population ($P < 0.05$) (Fig. 4.6 B). This population was significantly higher in the ED18-0 mM FA group combination than any other combination ($P < 0.05$). The IgM^{med} cell population was significantly lower in cells originating from ED15 embryos compared to all other combinations ($P < 0.05$). A significant interaction is also seen between age and FA for the IgM^{high} cell population ($P < 0.05$) (Fig. 4.6 C). This population was significantly lower in the ED21 embryos regardless of treatment. The main effect of embryo age can be observed here as well, with the IgM^{high} population rising with age ($P < 0.05$).

Table 4.4: Effect of FA on cell viability according to embryonic age

Status	Embryo age (days)			FA (mM)			SEM	P values		
	15	18	21	0	1.72	3.96		ED	FA	Age X FA
Live	92.04[(a)]	97.55[(b)]	98.63[(c)]	96.11	96.29	96.32	0.19	< 0.001	0.529	0.965

Values represent means of percent of cell population fitting each category from three independent experiments. Cells from ED15, 18 and 21 embryos were incubated with 0, 1.72 or 3.96 mM FA. Means sharing no common superscript letter differ significantly. Significant differences ($P < 0.05$) between means were tested post hoc by Tukey's test.

4.4.8 Effect of FA on staining embryonic B cell populations for both surface IgM and Propidium Iodide

The effect of FA on double stained cell populations according to age are presented in Table 4.6. Embryo age had a significant effect on all cell populations ($P < 0.05$). The dead $IgM^{low/med}$ cell population significantly decreased from ED15 to ED18 ($P < 0.005$). The population of dead IgM^{high} cells significantly decreased with age ($P < 0.005$), while the fraction of the population of live $IgM^{low/med}$ cells significantly increased with age ($P < 0.005$). For the live IgM^{high} cell population an interaction between age and FA was observed (Fig. 4.7) ($P < 0.05$). An ED21-0 mM FA group combination exhibited the lowest population of this kind compared to any other combination except the ED21-1.72 mM FA combination. The effect of embryo age is observed, as this population was significantly higher at ED15 then both of the other examined ages.

4.4.9 Effect of incubation time on embryonic chicken B cell RFC, TLR2b, Igβ and MHCII β chain mRNA gene expression by age

The effect of incubation time at different embryo ages on the mRNA levels of the genes of interest are presented in Figure 4.8. TLR2b expression was significantly downregulated in cells taken from ED18 and ED21 embryos after 4 hours compared to their expression at these ages with no incubation (control) (Fig. 4.8 A) ($P < 0.05$). At ED15 expression of Igβ was upregulated after 4 hours of incubation compared to control (Fig. 4.8 B) ($P < 0.05$). Four hours incubation caused a downregulation in RFC (Fig. 4.8 C) ($P < 0.05$) and MHCII β (Fig. 4.8 D) ($P < 0.05$) chain expression in cells from ED21 embryos.

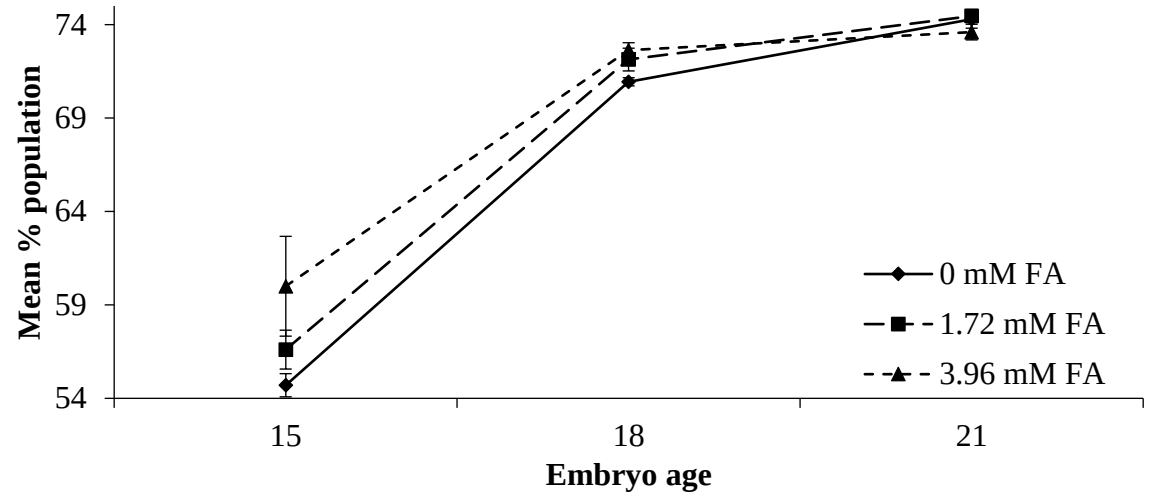
Table 4.5: Effect of FA on cell surface IgM expression according to embryonic age

Population	Embryo age (days)			FA concentration (mM)			SEM	P values		
	15	18	21	0	1.72	3.96		ED	FA	EDXFA
IgM^{low}	$57.27^{(a)}$	$71.91^{(b)}$	$74.12^{(c)}$	$68.57^{(a)}$	$69.37^{(ab)}$	$69.76^{(b)}$	0.19	<0.001	0.03	0.023
IgM^{med}	$19.13^{(a)}$	$21.72^{(b)}$	$22.37^{(c)}$	$21.8^{(a)}$	$20.89^{(b)}$	$20.42^{(c)}$	0.06	<0.001	<0.001	<0.001
IgM^{high}	$23.749^{(a)}$	$5.74^{(b)}$	$4.13^{(b)}$	$6.94^{(a)}$	$7.24^{(ab)}$	$7.59^{(b)}$	0.12	<0.001	0.044	<0.001

Values represent means of percent of cell population fitting each category from three independent experiments. Cells from ED15, 18 and 21 embryos were incubated with 0, 1.72 or 3.96 mM FA. Means sharing no common superscript letter differ significantly. Significant differences ($P < 0.05$) between means were tested post hoc by Tukey's test

Figure 4.6 Interaction plot for FA and embryo age effect on BCR expressing cell populations

A



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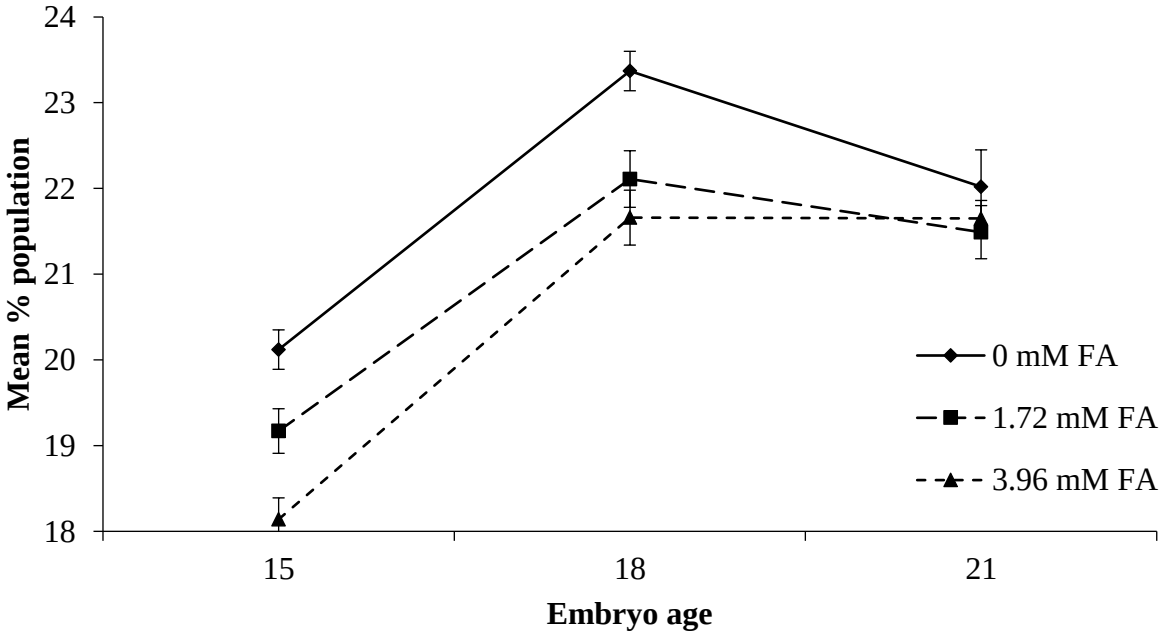

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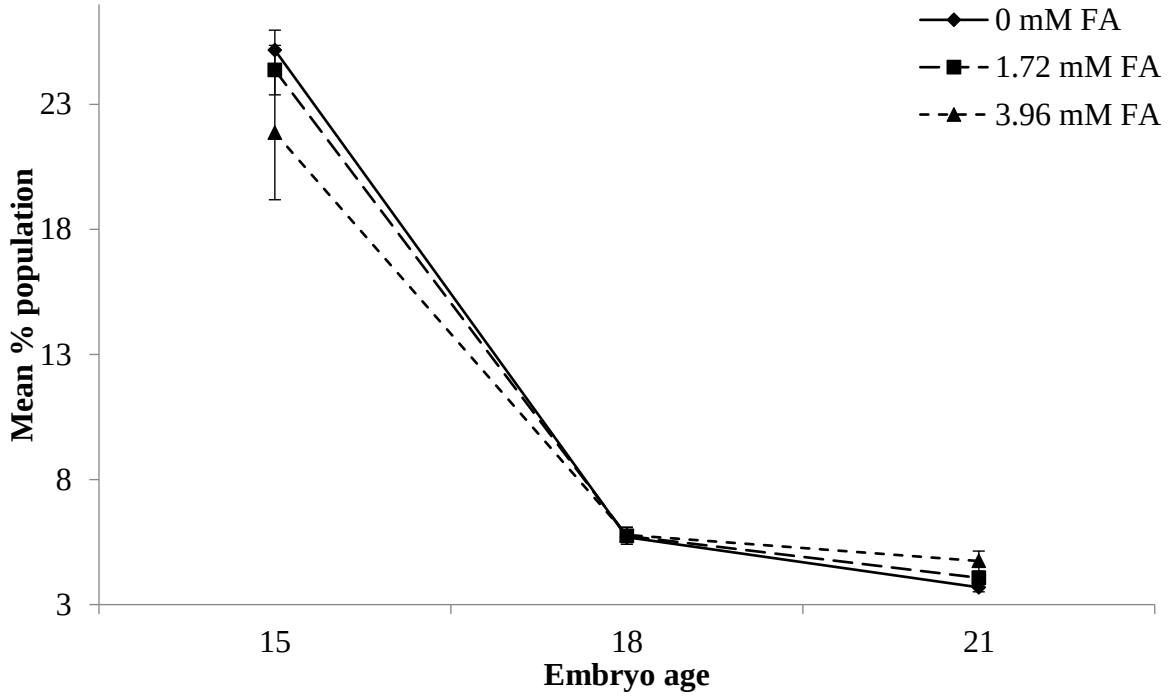


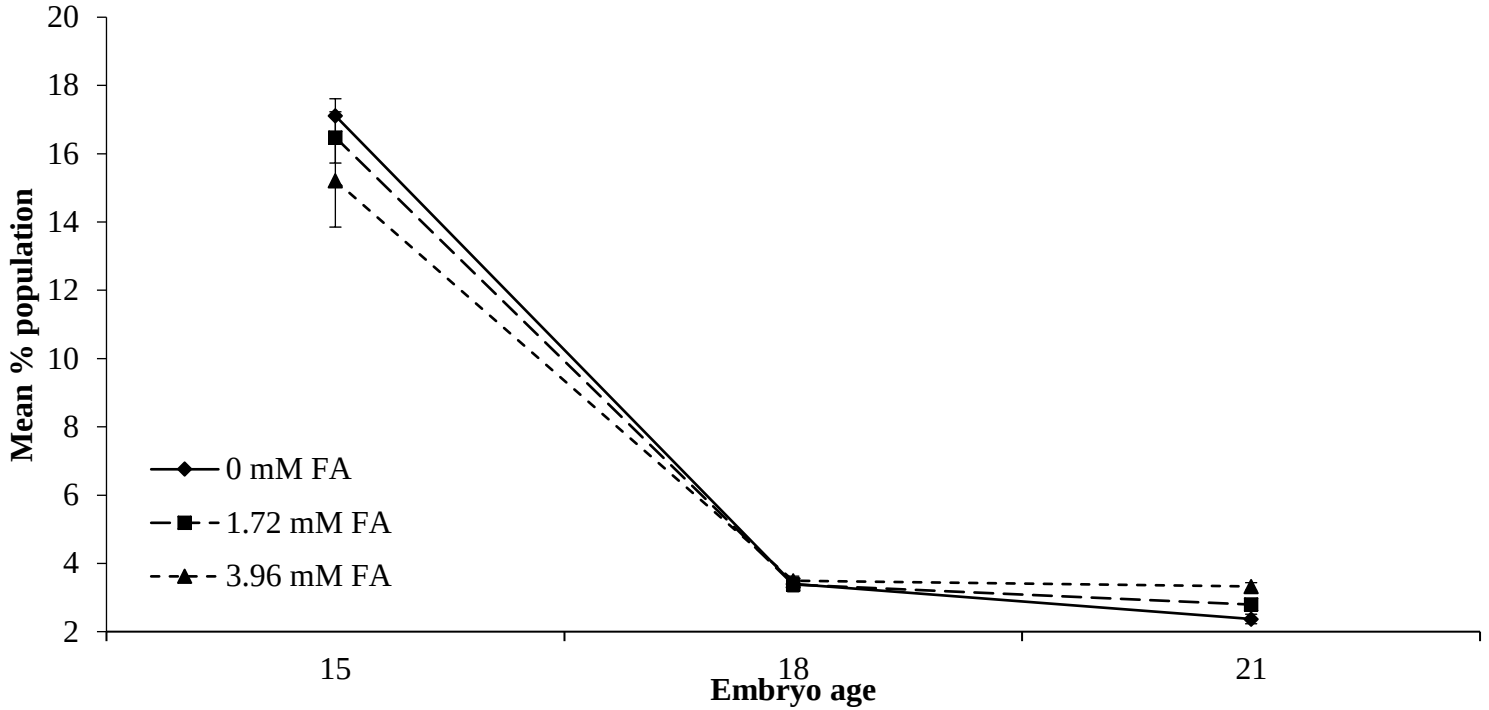
Points represent mean of % of population from three independent experiments repeated three times. The graphs represent the effect of age and FA interactions on (A) IgM^{low}, (B) IgM^{med} and (C) IgM^{high} populations. Lines represent SE. Significant differences between age and FA combinations were tested post hoc by Tukey's test. Differences between combinations in each cell population are significant at ($P < 0.05$).

Table 4.6: Effect of FA on staining embryonic B cell populations for both surface IgM and PI according to embryonic age

Population	Embryo age (days)			FA concentration (mM)			SEM	P values		
	15	18	21	0	1.72	3.96		ED	FA	ED X FA
Dead $IgM^{low/med}$	$0.48^{(a)}$	$0.23^{(b)}$	$0.21^{(b)}$	0.33	0.27	0.26	0.02	< 0.001	0.307	0.841
Dead IgM^{high}	$7.15^{(a)}$	$2.29^{(b)}$	$1.32^{(c)}$	2.53	2.43	2.46	0.09	< 0.001	0.852	0.496
Live $IgM^{low/med}$	$74.61^{(a)}$	$94.03^{(b)}$	$95.63^{(c)}$	90.08	90.15	89.6	0.28	< 0.001	0.166	0.351
Live IgM^{high}	$16.24^{(a)}$	$3.42^{(b)}$	$2.79^{(c)}$	$4.38^{(a)}$	$4.67^{(ab)}$	$5.02^{(b)}$	0.09	< 0.001	0.007	0.001

Values represent means of percent of cells found in each quadrant from three independent experiments. Cells from ED15, 18 and 21 embryos were incubated with 0, 1.72 or 3.96 mM FA. Means sharing no common superscript letter differ significantly. Significant differences ($P < 0.05$) between means were tested post hoc by Tukey's test.

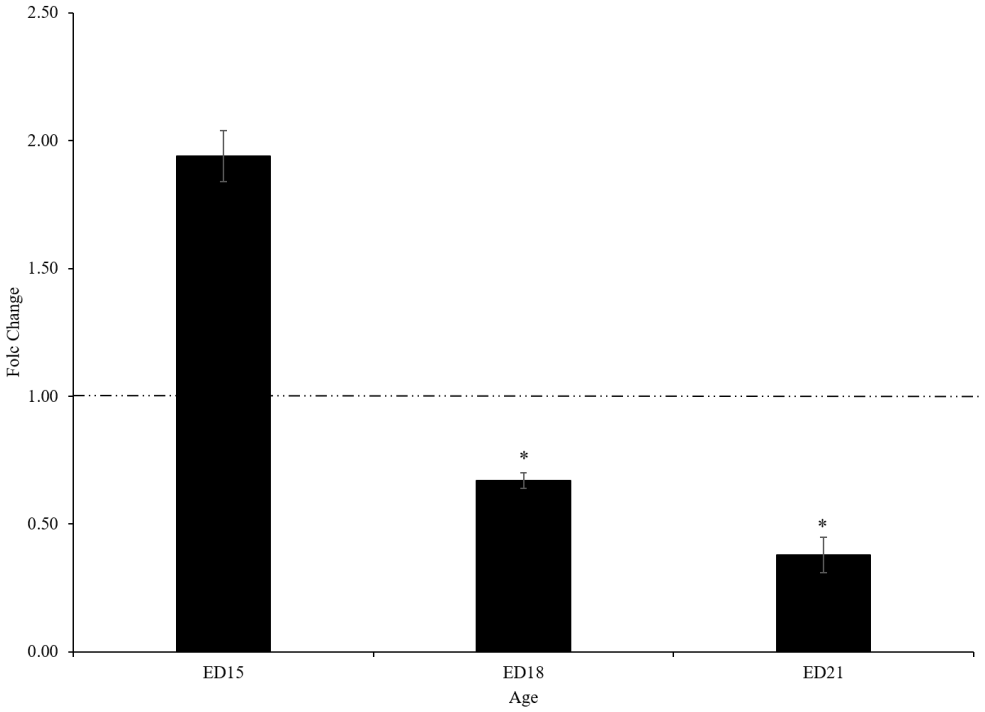
Figure 4.7 Interaction plot for FA and embryo age effect on live IgM^{high} cell population



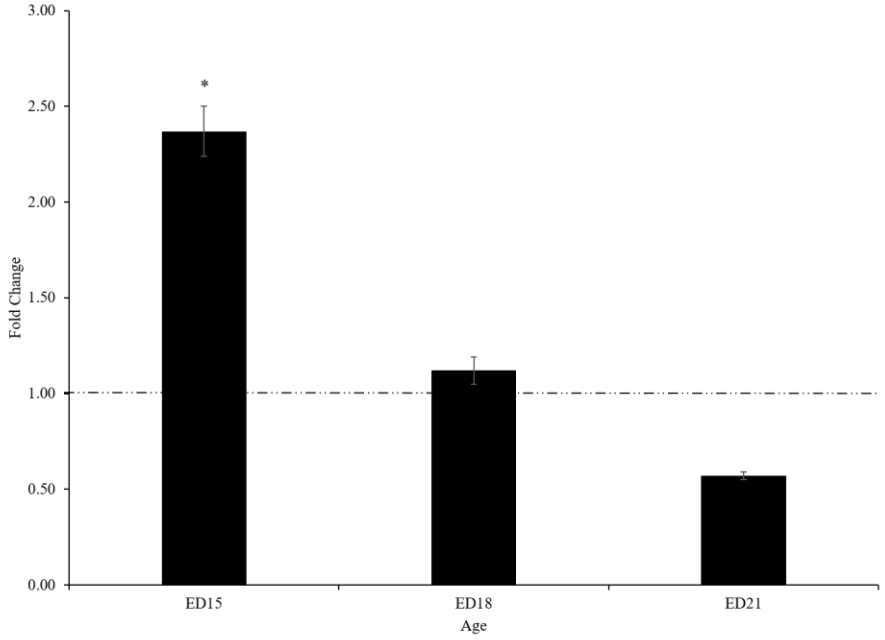
Points represent means from three independent experiments repeated three times. The graphs represent the effect of age and FA interactions on live IgM^{high} cell population. Lines represent SE. Significant differences between age and FA combinations were tested post hoc by Tukey's test. Differences between combinations are significant at ($P < 0.05$).

Figure 4.8 Effect of incubation time on TLR2b, Igβ, RFC and MHCIIβ chain mRNA gene expression

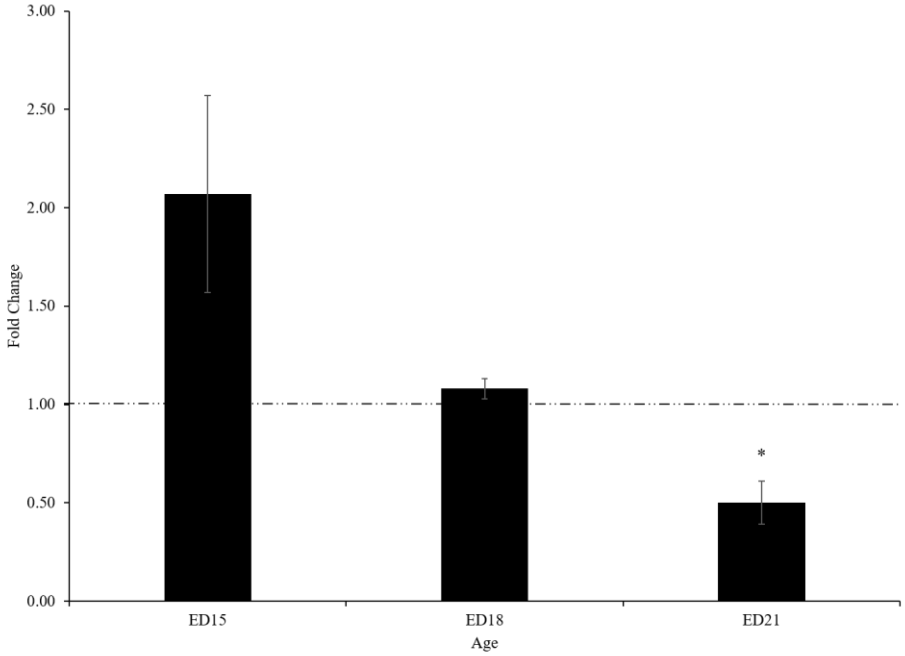
A



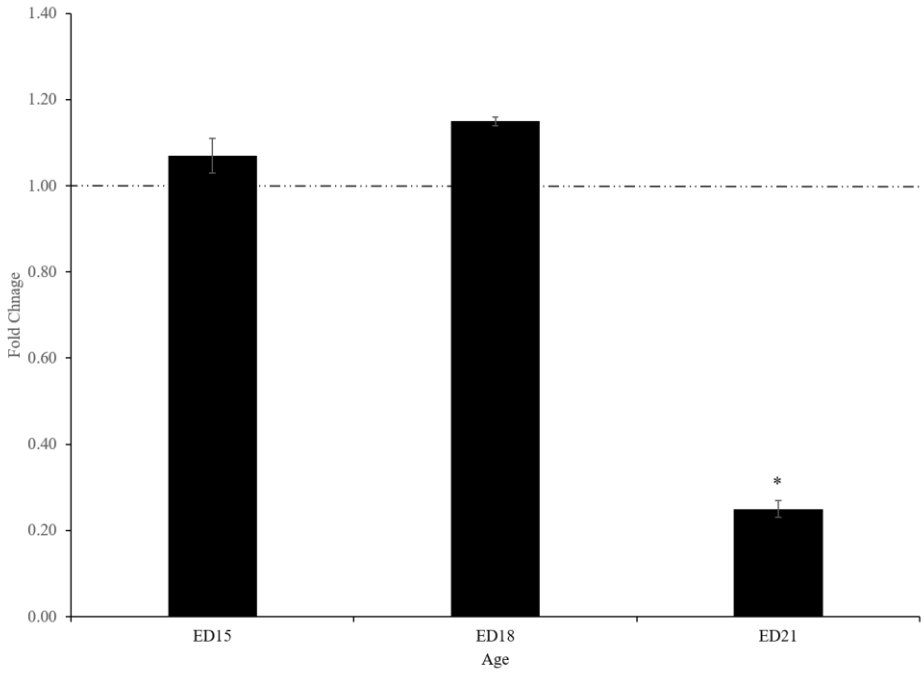
B


3.00
2.50
2.00
1.50
1.00
0.50
0.00
Fold Change
*
ED15
ED18
ED21
Age

C


3.00
2.50
2.00
1.50
1.00
0.50
0.00
Fold Change
*
ED15
ED18
ED21
Age

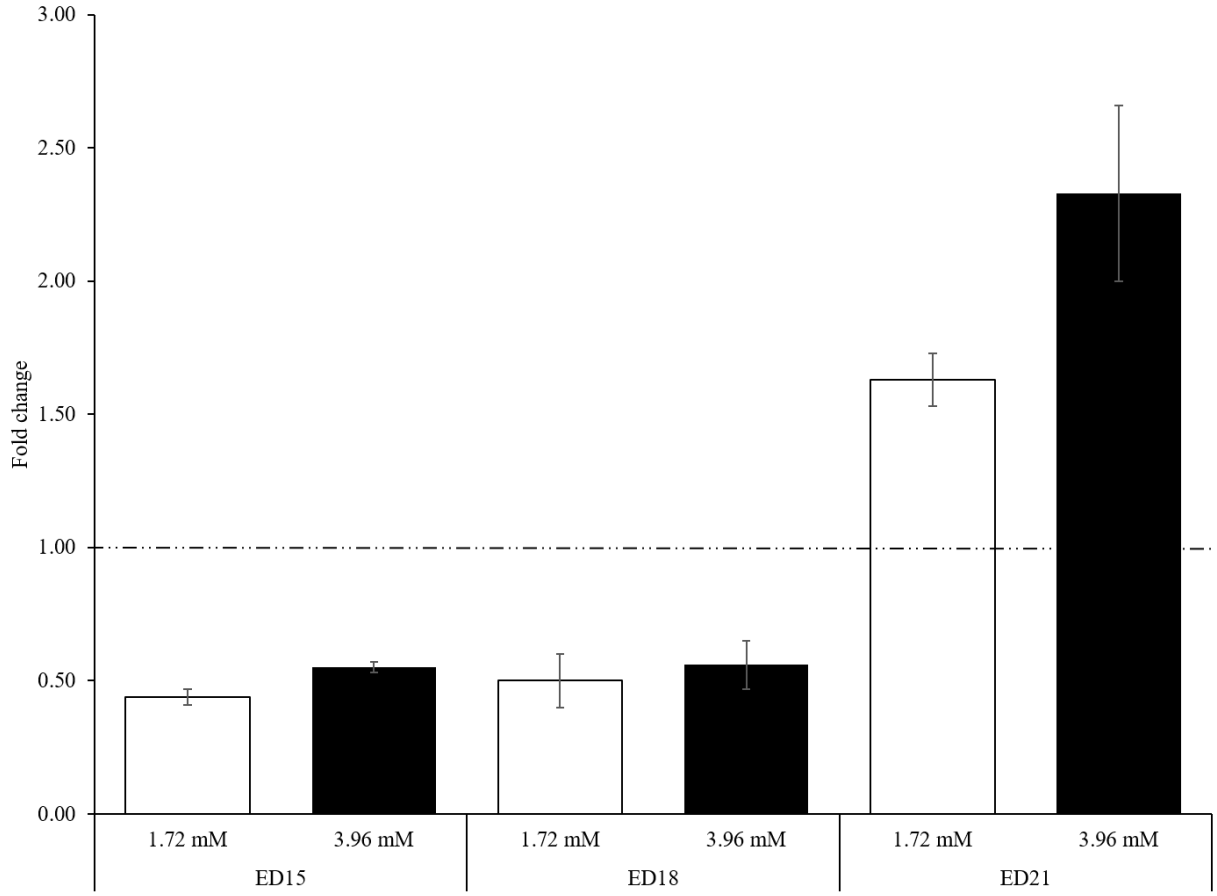
D



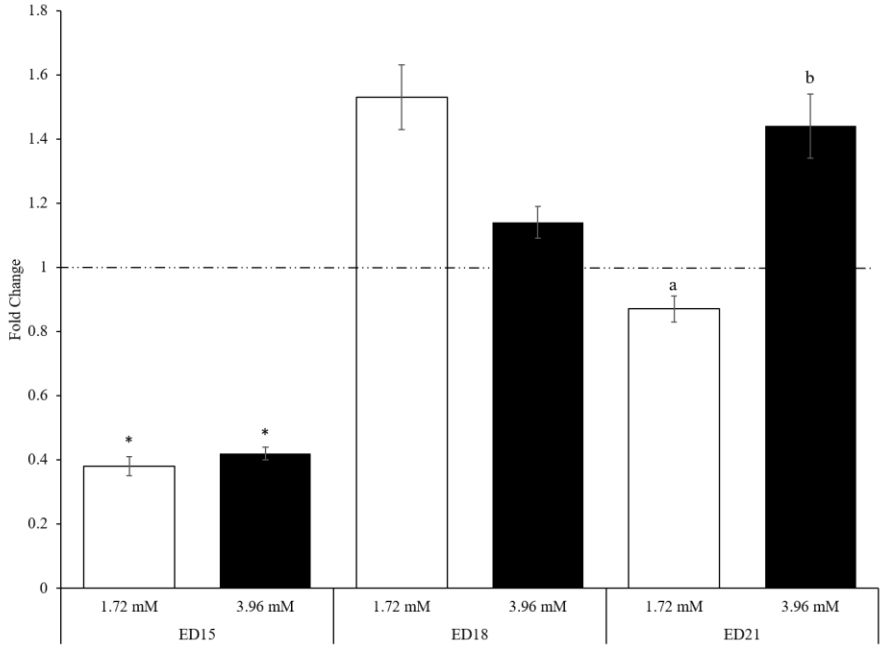
Fold expression of A) TLR2b, B) Igβ, C) RFC, and D) MHCII β chain after 4 hours of incubation. at ED15, ED18 and ED21 normalized against β actin expression. Values shown are means and SEM of triplicates of three independent experiments. Bars with * are significantly different compared to control (No incubation, dotted line) ($P < 0.05$).

Figure 4.9 Effect of FA on TLR2b, Igβ, RFC and MHCIIβ chain mRNA gene expression

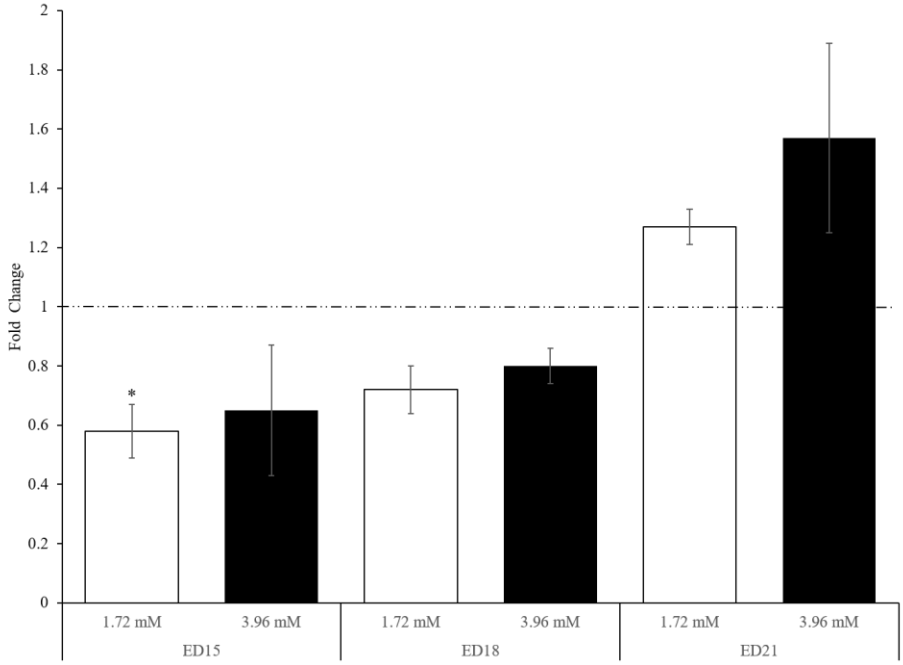
A



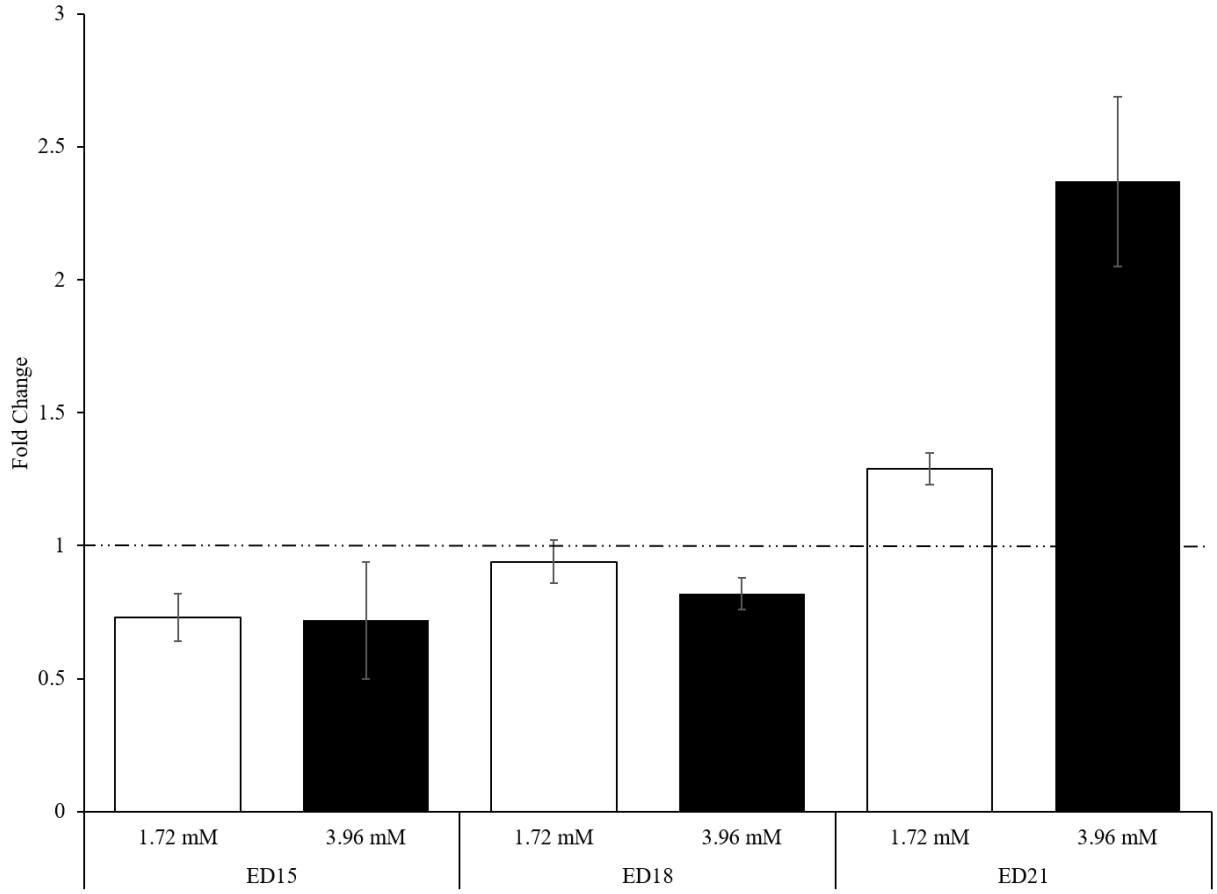
B


1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
Fold Change
*
*
a
b
1.72 mM
3.96 mM
1.72 mM
3.96 mM
1.72 mM
3.96 mM
ED15
ED18
ED21

C


2
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
Fold Change
*
1.72 mM
3.96 mM
1.72 mM
3.96 mM
1.72 mM
3.96 mM
ED15
ED18
ED21

D



Fold expression of A) TLR2b, B) Igβ, C) RFC, and D) MHCII β chain after treatment with 1.72 and 3.96 mM FA at ED15, ED18 and ED21 normalized against β actin expression. Values shown are means and SEM of triplicates of three independent experiments. Bars with * are significantly different compared to control (0 mM FA treatment for cells from each age, dotted line) ($P < 0.05$). Bars with different letters differ significantly between treatments within age group ($P < 0.05$).

4.4.10 Effect of FA on RFC, TLR2b, Igβ and MHCII β chain mRNA gene expression at different embryo ages

The effect of FA on the mRNA levels of the genes of interest at different embryo ages are presented in Figure 4.9. TLR2b mRNA levels were not significantly different from those demonstrated in the control group regardless of treatment or age (Fig. 4.9 A) ($P > 0.05$). At ED15 incubation with 1.72 mM FA or 3.96 mM FA resulted in a significant downregulation of Igβ ($P < 0.05$). While the FA treatments did not result in significant change in Igβ expression when compared to the expression of the same gene in the control group, there was a significant difference in the expression of this gene between the cells treated with 1.72 mM FA and those treated with 3.96 mM FA ($P < 0.05$) (Fig. 4.9 B). RFC expression was downregualtred in cells extracted from ED18 embryos (Fig. 4.9 C). FA did not have a significant effect on MHCII β chain expression at any age ($P > 0.05$) (Fig. 4.9 D).

4.4.11 Effect of incubation time on the methylation profile of TLR2b, Igβ and MHCII β chain

The methylation profile of each promoter region at ED15, ED18 and ED21 after 0 h of incubation and after 4 hours of incubation is presented in Figure 4.10. The proximal promoter region of TLR2b remained 100% methylated regardless of age or incubation time except for one replicate from ED15 taken from cells incubated for 4 hours (Fig. 4.10 A). At ED15, the Igβ promoter region was hypermethylated, with 100% promoter methylation after 0 h incubation except for one unmethylated CpG in one replicate after 4 hours. At ED18 a 100% methylation observed after 4 hours, except for one unmethylated CpG in one replicate from the 0 h incubation group. At ED21,

the promoter region of Igβ is hypomethylated, with only one dinucleotide methylated in one replicate from the 0 h cell group, and a completely unmethylated promoter in the other two replicates. After 4 hours, the promoter region remains hypomethylated, with 2 CpGs methylated in one replicate, and no methylation seen in the other two (Fig. 4.10 B). The MHCII β chain promoter region was completely unmethylated in all replicates of the 0 h incubation treatment group at all three time points, and remained completely unmethylated after 4 hour when the cells originated from ED18 embryos. The same can be seen in two replicates out of three for ED15 and ED21 cells after 4 hours of incubation. Three dinucleotides were methylated in the remaining replicate at ED15 and 8 CpGs were methylated in the remaining replicate at ED21 (Fig. 4.10 C).

4.4.12 Effect of FA on the methylation profile of TLR2b, Igβ and MHCII β chain

The methylation profile of each promoter region at ED15, ED18 and ED21 following treatment with 0, 1.72 or 3.96 mM FA is presented in Figure 4.11. The TLR2b promoter region remained methylated regardless of age or FA treatment, except one replicate taken from cells treated with 0 mM FA at ED15 (Fig. 4.11 A). The Igβ promoter region remained hypermethylated at ED15 and ED18, demonstrating a 100% of CpGs methylated in all replicates regardless of treatment, except one replicate at ED15 taken from cells treated with 0 mM FA, where one dinucleotide was unmethylated. At ED21, the promoter region is hypomethylated, as all CpG dinucleotides are unmethylated except for one replicate taken from 0 mM FA treated cells, where 2 dinucleotides were methylated (Fig. 4.11 B). The MHCII β chain promoter region demonstrated hypomethylation throughout treatments and ages, with no methylation observed in the sequences except three CpGs in one replicate taken from ED15 cells treated with 0 mM FA. The same

methylation level was seen in one replicate originating from ED21 cells treated with 3.96 mM FA, and 5 CpGs were methylated in one replicate taken from cells treated with 0 mM FA taken from that time point (Fig. 4.11 C).

4.4.13 Association between concentration of FA and percent of TLR2b, Igβ and MHCII β chain promoter methylation

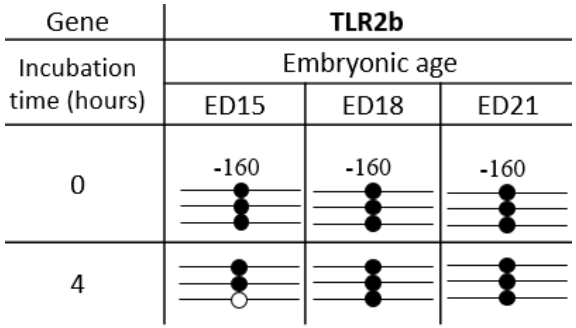
No association was found between the concentration of FA used in the treatments and the percent of promoter methylation of the TLR2b, Igβ and MHCII β chain promoters.

4.4.14 Association between percent of TLR2b, Igβ and MHCII β chain promoter methylation and gene expression

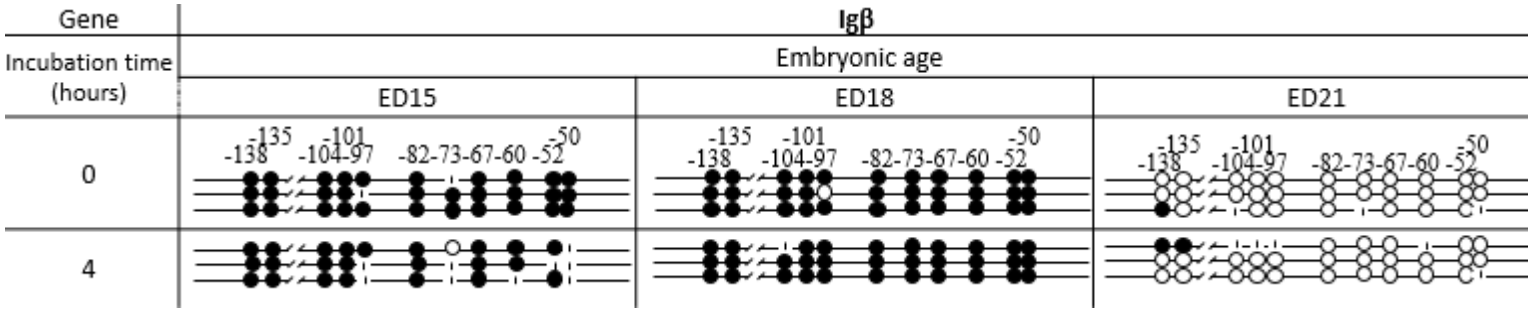
No association was found between the percent of promoter methylation of the TLR2b, Igβ and MHCII β chain promoters and the expression of the respective genes.

Figure 4.10 Incubation time effect on proximal promoter methylation profile of TLR2b, Igβ and MHCII β chain at ED15, ED18 and ED21

A



B



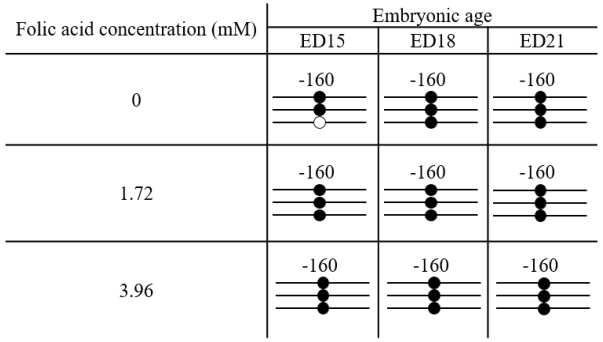
C

Gene	MHCII β chain		
Incubation time (hours)	Embryonic age		
	ED15	ED18	ED21
0	-167 -134 -101-94-88-82 -61-49 -40 -30 -14 -174-159 -128-108-98-91-85 -65-55-47 -38 -18-11	-167 -134 -101-94-88-82 -61-49 -40 -30 -14 -174-159 -128-108-98-91-85 -65-55-47 -38 -18-11	-167 -134 -101-94-88-82 -61-49 -40 -30 -14 -174-159 -128-108-98-91-85 -65-55-47 -38 -18-11
4			

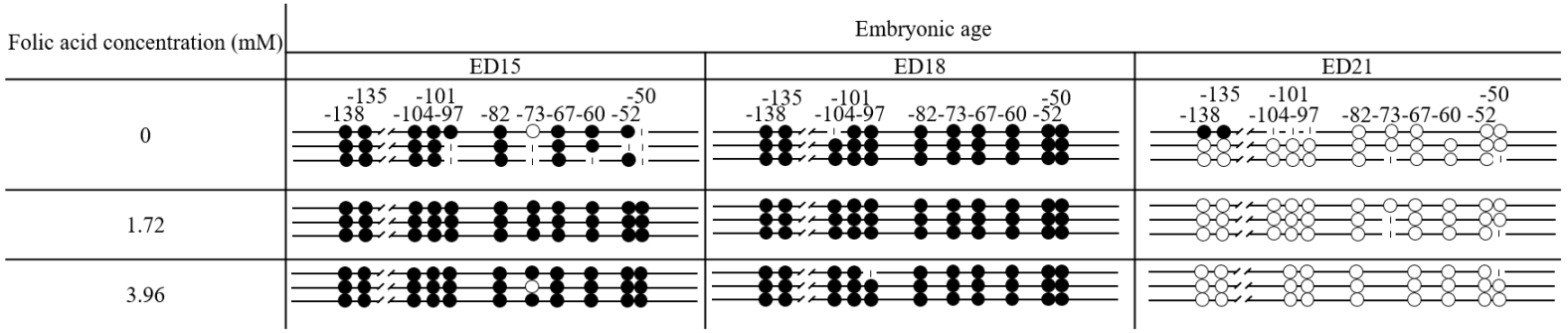
Sequence analysis following a bislufite conversion of the proximal promoter region (200 bp upstream of the start codon) after no incubation (0 h) and 4 hours incubation (4 h) of (A) - TLR2b, (B) - Igβ and (C) - MHCII β chain at ED15, ED18 and ED21. Each treatment was triplicated and each replicate converted, amplified and sequenced independently. (○) - unmethylated CpG dinucleotide (●) - methylated CpG dinucleotide. Numerical values represent the location of the cytosine relative to the start codon. A break in the line indicates a sequence of at least 15 base pairs without CpG dinucleotides.

Figure 4.11 FA effect on the proximal promoter methylation profile of TLR2b, Igβ and MHCII β chain at ED15, ED18 and ED21

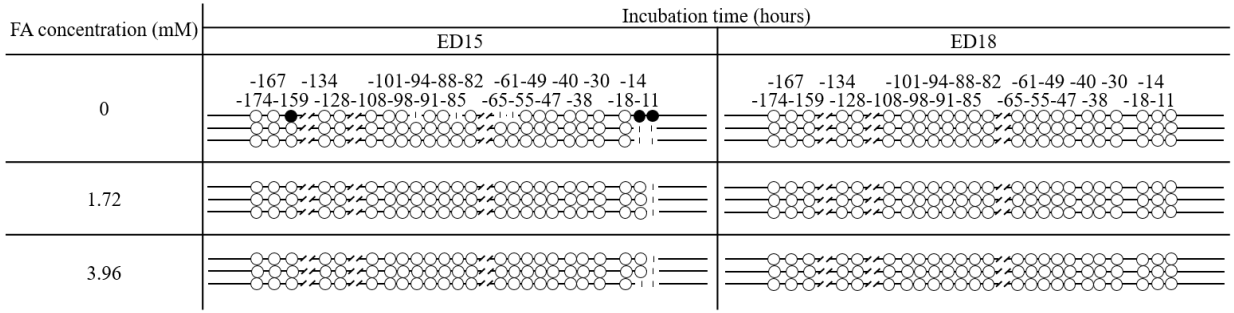
A

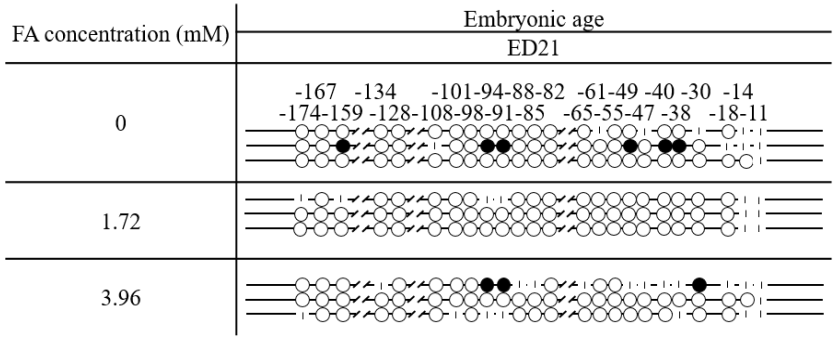


B



C





Sequence analysis following a bislufite conversion of the proximal promoter region (200 bp upstream of the start codon) after treatment with either 0, 1.72 or 3.96 mM FA of (A) - TLR2b, (B) - Igβ and (C) - MHCII β chain at ED15, ED18 and ED21. Each treatment was triplicated and each replicate converted, amplified and sequenced independently. (○) - unmethylated CpG dinucleotide (●) - methylated CpG dinucleotides Numerical values represent the location of the cytosine relative to the start codon. A break in the line indicates a sequence of at least 15 base pairs without CpG dinucleotides.

4.5 Discussion

The goal of this study was to determine the effects of FA on the ability of the chicken B cell to function as an immune cell by examining its effect on TLR2b, Igβ and MHCII β chain. The *ex-vivo* model chosen presented a problem, B cells extracted from the BoF had a significant loss in viability, which was also reported elsewhere (Compton, M.M. and Waldrip, H.M. 1998, Luna-Acosta, J.L. et al. 2015). In these studies cell viability declined to 63% after only 4 hours. In the current study the addition of 5% of chicken serum to the growth media generated viabilities above 90% for the duration of the experiment (4 hours) (data not shown). The effect is evident when the viability of the cells was tested using PI staining in flow cytometry. Cells originating from ED18 and ED21 embryos showed no significant loss of viability when not incubated or after 4 hour incubation. The population of viable cells harvested from ED15 embryos was significantly lower than the initial 0 hour populations of the other two time periods. At ED15 the migration of pre-B cells to the BoF has just ended. However, the distinct cortical and medullary areas of each follicle are not fully developed by this time (Funk, P.E. and Palmer, J.L. 2003). It is possible that the trypsin treatment used to extract the cells damaged the target cells of this experiment, which would explain the lower population of viable cells at ED15 when the cells were not incubated. This time point was also the only one during which a significant difference was observed between the viability of cells in the T(0) group and the T(4) group. The BoF expresses several compounds that limit apoptosis during development, such as Survivin (Johnson, A.L. et al. 2002), growth hormone (Luna-Acosta, J.L. et al. 2015), B cell activating factor (BAFF) (Koskela, K. et al. 2004) and Blimp1 (Wan, Z. et al. 2014). At least one of which (Blimp-1) is expressed up to ED18 and then

downregulated (Wan, Z. et al. 2014). Furthermore, interactions between developing chicken B cells and the bursal epithelium has also been shown to inhibit apoptosis (Funk, P.E. and Thompson, C.B. 1998). It has also been demonstrated that as B cell development progresses, they lose expression of some of their membrane proteins such as CD38, CD10 and CD39 (Thellin, O. et al. 1998). Taken together, we can infer that B cells from ED15 embryos were more dependent on anti-apoptotic signals in the BoF, and thus removing them from that environment resulted in greater death of these cells than that observed in cells extracted from ED18 and ED21 embryos.

There are several factors that may affect the amount of BCR expressed on the surface of the cells during the chicken B cell development process. Heavy and light chain rearrangement, a process that occurs in pre B cells before they immigrate to the BoF (Reynaud, C.A. et al. 1991) and during the first days after immigration, can result in cells with non-functional BCRs. However, these cells are not selected for a productive BCR, and they will go through another step in antibody diversification - gene conversion, taking place while the cells rapidly proliferate in the follicles (Reynolds, J.D. 1987). During this process, B cells that are unable to initiate a signaling cascade via Igα and Igβ, are autoreactive (Davani, D. et al. 2014a) or are unable to produce a functional BCR in the conversion process go through apoptosis (Sayegh, C.E. et al. 1999). However, before this process is initiated, a downregulation of surface BCR is observed (Paramithiotis, E. et al. 1995). The results of this study are in agreement with this observation, as an increase in the IgM^{low} population was observed between ED15, the end of the migration wave, and ED18, when cells start to emigrate out of the BoF and colonize secondary lymphoid tissue (Davani, D. et al. 2014b).

The role of the BoF in maintaining these cells can be observed here as well, as a significant reduction in the IgM^{low} population can be seen when ED15 cells were incubated outside the BoF for 4 hours. In later stages of B cell development cells expressing low levels of IgM will go through apoptosis (Paramithiotis, E. et al. 1995), a fact that can be seen by the significant reduction in this population between ED18 and ED21. It is possible that removing the cells from the bursal environment resulted in removal of the pro-apoptotic signal, which could account for the observed significant increase in this population between 0 hours and 4 hours of incubation at ED21. While the interaction between the BoF and IgM^{low} cells between ED18 and ED21 results in reduction of this population, the opposite was observed for the IgM^{med} population. When a distinction was made between live and dead $IgM^{low/med}$ cells, the portion of dead cells was observed to be significantly lower than any other population, remaining below 1% regardless of age or incubation time. For that reason, the changes seen in the live IgM $^{low/med}$ are the sum effect of both IgM^{low} and IgM^{med} populations. For example, a significant rise of IgM^{low} cells between ED15 and ED18, with no change in the IgM^{med} population was observed as a rise in live $IgM^{low/med}$ cells between those time points. The observed upregulation in live $IgM^{low/med}$ cells between ED15 and ED18 after 4 hours of incubation out of the BoF is in accordance with the raise of both IgM^{low} and IgM^{med} populations under the same conditions. While in the BoF, this population is signaled to proliferate rather than die. That signal appears to be lost once the cells are removed from the BoF. It is possible that these IgM^{med} cells are signaled to proliferate as they will become IgM^{high} cells that are ready to colonize the periphery. This notion is strengthened by the fact that between ED18 and ED21 there is no significant change in the population of live $IgM^{low/med}$ cells, as they act as a reservoir for the IgM^{high} population. The IgM^{high} population at ED15, like the other IgM expressing populations, is the most

sensitive to environmental change compared to the same population at ED18 and ED21. However, harvesting these cells has the opposite effect compared to the other two populations. i.e., incubating the cells outside of the bursal environment resulted in a higher percentage of this population at ED15. Since the gene expression assays were done on the general cell population for each examined age, we cannot distinguish whether the Igβ upregulation observed at ED15 happens solely in the IgM^{high} population. However, it is tempting to pair the upregulation of this gene with the increase in the IgM^{high} population, as the effect of 4 hours incubation on ED15 IgM^{low} and IgM^{med} populations was inhibitory. This could possibly indicate the presence of either a compound in the chicken serum that induces the expression of the BCR complex, a BCR inhibitory compound in the BoF, or both. However, to the best of our knowledge no research has been published on the subject. As seen before, cells from ED18 and ED21 are less sensitive to the environmental change, and the ratio of IgM^{high} cells remains steady during these days. The IgM^{high} population is committed to peripheral and secondary lymphoid tissue colonization (Narabara, K. et al. 2009). Interestingly, both live and dead populations of IgM^{high} increased when ED15 cells were incubated for 4 hours. As mentioned before, the increase in live cells was concurrent with a rise in Igβ expression. The rise in dead cells, though significant, was small (4.7%), and it is possible that the proportion of these type of cells increased as the proportion of live $IgM^{low/med}$ decreased. No significant difference was found between the populations of live IgM^{high} cells between ED18 and ED21, and the same is true for the dead IgM^{high} population. As B cells start to emigrate out of the BoF around ED18 (Davani, D. et al. 2014b), it is possible that an equilibrium between proliferation and apoptosis processes keeps these populations stable between the start of emigration and day of hatch. It has been previously described that the vast majority (95%) of B cells in the BoF die *in*

situ, while only the remaining 5% emigrate out of the BoF and colonize the periphery (Paramithiotis, E. and Ratcliffe, M.J. 1993). Although the proportion of live IgM^{high} we observed at ED21 was 1.5%, it points towards the same conclusion - The B cell population in the BoF is heavily selected against before hatch, with most of the population not surviving the process.

While the effect of incubation time on Igβ expression at ED15 is discussed above, the changes in RFC, TLR2b and the MHCII β chain were also examined at all time points under these conditions. At ED18, a downregulation of TLR2b was observed. There is no published data on the role of TLR2b in the BoF, or in B cell maturation in the chicken or any other animal, and for this reason, it is hard to confirm whether the observed downregulation is due to the removal of the cells from the BoF or because there is an inhibitory signal for TLR2b expression in the chicken serum, or both. Because of the lack of knowledge on this subject, it is hard to conclude whether this downregulation has any biological significance, and needs to be further studied. At ED21 a downregulation of TLR2b was also observed. These changes in gene expression were not associated with any changes in the methylation of the promoter region of the TLR2b gene, which remained methylated throughout all experiments. A likely conclusion would be that the expression of the TLR2b gene is not controlled by proximal promoter DNA methylation. At time of hatch, ED21, a significant downregulation of the reduced folate carrier (RFC) and the MHCII β chain was observed as well. These changes in MHCII expression were found not to be associated with changes in the methylation profile of its promoter, as the region remained hypomethylated regardless of age or incubation time. These results are in agreement with others who found no association between the methylation status of this gene and its expression (Levine, F. and Pious, D. 1985, Yuan, X.J. et al. 1994). Igβ appeared lower compared to control, however that change

was not significant. The methylation status of the Igβ promoter region was the only one that changed during these experiments. It was completely hypermethylated at ED15 and ED18 cells, but hypomethylated at ED21 regardless of incubation time. These results indicate that removing the cells out of the BoF for 4 hours does not affect the methylation profile of the proximal promoter regions of the genes of interest.

While placing cells in a culture has been shown to affect DNA methylation, the shortest period of time of incubation that resulted in such changes is three days (Nestor, C.E. et al. 2015). It is possible that the control over the expression of the examined genes is not epigenetic. In mice a transcription factor, MARCH-1, was found expressed by mature B cells, where it downregulated the expression of MHCII (Matsuki, Y. et al. 2007). In the same animal it was also found that mature and resting B cells express less surface IgM than immature or transitional ones (Carsetti, R. et al. 1995). However, this has not been studied in chickens and the nature of these BoF dependent gene upregulations, their downregulation outside of the bursal environment, or indeed the existence of both signals, remains to be elucidated.

Observations of the viability of the cells when those are removed from the BoF and cultured *ex vivo* permitted the observation of the effect of FA treatment on the developing chicken B cells, as above 90% of cells remained viable after the incubation period. Our results show no significant effect of FA treatment on cell viability. Most work on the relationship between FA and cell viability describe only FA deficiency as an affecting factor (Courtemanche, C. et al. 2004, Moussa, C. et al. 2015). This effect is more likely related to the role FA has in DNA synthesis, rather than methylation, as it was previously described that FA deficiency results in an increase in DNA

instability, including breakage and defective repair (Duthie, S.J. and Hawdon, A. 1998). The effect of FA on cell viability (Williams, P.J. et al. 2011, Ahmed, T. et al. 2016) is an increased apoptotic rate in cells treated with FA concentrations one hundred times higher than physiological levels (Williams, P.J. et al. 2011). The growth media that was used in our experiments contained a basal amount of FA (0.008 mM), which suggests that the cells were not FA deficient throughout the experiment. Furthermore, the concentrations of FA added to the bursal cells are the ones found in the yolk sac when the hens were fed a normal NRC approved diet (1.72 mM FA) or the maximal amount of FA found in these animals after the diet was enriched with FA (3.96 mM FA). The latter concentration represents yolk sac saturation levels, as adding more it to the diet did not increase FA in the yolk sac (House, J.D. et al. 2002). Using these concentrations meant that the cells were not exposed to excessive or supraphysiological amounts of FA, which would explain the lack of effect of FA on cell viability observed in this study.

The lack of interactions between FA concentration and age allows better observation on the effect of age on cell viability. More viable cells were found as embryo age progressed. It is possible to infer that FA concentration in the yolk sac, achieved through a normal or an enriched diet, will not have an effect on the number of B cells proliferating in the embryonic BoF.

An interaction between embryonic age and FA concentration was observed affecting BCR levels on the cells surface. Pairwise comparison has shown that there was no significant difference between treatments for each age tested. Furthermore, when the cells were treated with either 0 or 1.72 mM FA the number of IgM^{low} cells rose as age progressed. However, the population of IgM^{low} cells from ED18 and ED21 that was treated with 3.96 mM FA was not significantly different

(although both were higher than the IgM^{low} ED15 cell population). The behavior of the IgM^{low} cell populations is comparable to that seen for IgM^{low} cells incubated for 4 hours out of the BoF. However, it seems that treatment with 3.96 mM FA halted the proliferation of these cells between ED18 and ED21. Inhibition of cell proliferation by FA has been described before (Carmody, B.J. et al. 1999, Jaszewski, R. et al. 1999). However, we demonstrated that while in the BoF the IgM^{low} cell population is reduced between ED18 and ED21. This effect of 3.96 mM FA on IgM^{low} cells, while interesting, does not have a physiological relevance. The effect of FA is most visible on IgM^{med} cells. At ED15, this population was significantly lower when the cells were treated with 3.96 mM FA. At ED18, treatment with both 1.72 mM FA and 3.96 mM FA resulted in significantly lower populations of IgM^{med} compared to the 0 FA group. At ED21 there was no significant difference between the populations of IgM^{med} cells.

One of the stages in which FA is utilized in the DNA synthesis pathway is when the cells are replicating. In a process of maintenance methylation, the enzyme DNMT1 uses the methylated mother DNA strand as a template for the methylation of the daughter strand (Ghoshal, K. and Bai, S. 2007). The fact that the IgM^{med} population was the most responsive to FA treatment strengthens the notion that these cells are the ones to proliferate and act as a reservoir for the IgM^{high} B cells that will emigrate out of the BoF. In fact, we demonstrated that while in the BoF, this population increases from ED18 to ED21, an effect that is not observed in any of the groups incubated out of the BoF for 4 hours. It is possible that FA treatment applied directly to the BoF will reduce the proliferation of the IgM^{med} cell population, which in turn might affect the IgM^{high} population leaving the BoF, however this needs to be studied further.

As with the pairing of Igβ upregulation at ED15 with the increase of the IgM^{high} population at this time point at 4 hours of incubation, it cannot be determined in which population the observed downregulation of this gene occurred (the downregulation is significant when ED15 cells treated with either 1.72 or 3.96 mM FA). However, the ED15 IgM^{med} population is the only one that decreased with FA treatment. The only significant difference observed in the IgM^{high} population was when the cells originated from ED21 embryos. This population was significantly higher after the cells were treated with 3.96 mM FA compared to 0 FA treatment. Although significant, the change in population size is around 1% (3.7% of the population at 0 mM FA to 4.7% of the population at 3.96 mM FA), which leads us to conclude that FA treatment will not affect cells expressing high levels of IgM in a way that will affect the immune capabilities of the cell or the organism. The effect of age on each population observed was comparable to the effect of this factor when incubation time effect was examined. When a distinction was made between live and dead cells in each population, age had the same effect as the one observed in the incubation time experiments. The age effect was clearer in the FA experiments, as FA did not have an effect on dead or alive $IgM^{low/med}$ cells or on the dead IgM^{high} cells. The only population in which FA had an effect on and an interaction with the age factor was the live IgM^{high} population. The pairwise comparison gave identical results to those observed for the total IgM^{high} population. A single significant difference between the 0 mM FA and 3.96 mM FA groups harvested from ED21 embryos, with a 1% difference between the means of each group.

Treatments with FA were conducted to determine its effect on the expression of the genes that B cells rely on for their immunological functions. The downregulation of Igβ expression at ED15 was discussed above, and that was the only significant change observed at this age regardless of

treatment. Treatment with FA did not affect the mRNA levels of TLR2b, Igβ and MHCIIβ chain in ED18 and ED21 cells in a significant manner. Inspecting the methylation status of the promoter regions of these genes, there was no significant association observed between FA treatment and percent of methylation, and between the latter and gene expression. As observed in the comparison between cells that were not incubated and cells incubated for 4 hours, the TLR2b promoter region was hypermethylated regardless of FA treatment. This is true for the Igβ promoter region in ED15 and ED18 cells as well. Conversely, the Igβ promoter region at ED21, as well as the MHCII β promoter region at all examined ages remained hypomethylated regardless of FA treatment.

The results regarding the association between FA treatment and the promoter methylation of Igβ and MHCII β chain are contradictory to the results obtained when DT-40 cells were used. In these cells a significant association between these two factors was observed (Elad O. et. al., unpublished results). When examining the differences between the Igβ and MHCII β chain promoter methylation profile in DT-40 cell line and the primary cell culture, it appears that the CpG dinucleotides closer to the start codon are hypermethylated in the cell line, and hypomethylated in the primary cell culture. This result is in agreement with other publications, which found that cancer linked hypermethylation is concentrated around the transcription start site region, and can occur around genes that are not linked to cancer progression (Ehrlich, M. 2002).

Another discrepancy between the work done with DT-40 and the work done on primary chicken B cell cultures is that treating DT-40 cells with 1.72 mM FA resulted in significant upregulation of TLR2b, and a 3.96 mM FA treatment resulted in upregulation of MHCII β chain expression, while both genes were not significantly affected in primary B cells. According to the

manufacturer's information, DT-40 cells are prepared from 1 day old chicks. Looking at the effect FA had on ED21 cells (the closest date to 1 day old chicks examined), one can observe a trend of upregulation of both TLR2b and MHCII β chain as FA concentration increases. However, these changes are not statistically significant. C-rel and v-rel, two oncogenes inserted into the B cell as part of the production of DT-40 cells, are known to upregulate MHCII expression (Hrdlickova, R. et al. 1994). If this effect is cumulative or synergistic with the effect of FA on this gene, it could provide an explanation as to why the upregulation of the MHCII β chain gene was significant in the DT-40 cells and not the primary B cell culture. The uniqueness of the DT-40 cell line might also explain the upregulation of TLR2b. It was demonstrated that TLR2 can bind and be activated by viruses (Chen, S. et al. 2013). As DT-40 cells secrete low levels of infectious RAV-1, perhaps the difference in the significance of upregulation of TLR2b between the two cell types is due to recognition of RAV-1 by the DT-40 expressed TLR2b. To the best of our knowledge, no work has been published on the effect interaction between oncogenes and FA has on MHCII expression, as well as research about the recognition of RAV-1 through the chicken TLR2b.

At ED18, the RFC gene expression was downregulated when the cells were treated with 1.72 mM FA. It appears that this trend of downregulation continues when the cells were treated with 3.96 mM FA, however it was not significantly lower than the expression observed in the control group. Previous research has demonstrated that both the RFC and PCFT, the two main FA transporters, are downregulated when the cells that express them are exposed to FA. This reduction is observed both for mRNA as well as protein levels (Ashokkumar, B. et al. 2007). The effect of FA supplementation was also examined in chicken intestines where it caused an insignificant reduction in RFC mRNA levels in the jejunum (Jing, M. et al. 2009). The reason behind the

reduction in RFC expression being significant when the cells were treated with a low amount of FA, and insignificant when the higher concentration was used is unclear. However, the response to the treatment with FA at this time point is a reduction in mRNA levels of this gene, which is in agreement with the articles mentioned above.

4.6 Conclusions

A novel method by which chicken B cells extracted from embryonic BoF can be kept viable for longer periods of time than previously published is demonstrated in this work. This method can enhance research investigating the processes that chicken B cells go through while maturing. It can also be used to better understand the effect of compounds in the BoF on the developing B cell, at different stages of embryogenesis. By removing the B cells from the bursal environment, we have demonstrated the importance of the BoF in B cell development in both selection and proliferation. Treatment with FA, while not associated with direct changes to the proximal promoter region of TLR2b, Igβ and MHCII β chain, has affected the expression of Igβ at ED15. As Igβ is required in order to pass the selection process in the BoF, FA treatment might change the proportion of B cells that leave the BoF. It was demonstrated here that treatment with FA reduces the proportion of the population expressing medium levels of IgM, and that it is likely that the observed reduction in Igβ expression is paired with the reduction of this population, although this has to be further studied. Taken together, FA has been shown to have immunomodulatory properties affecting B cell development in the chicken embryo. Whether these changes affect the immune capabilities of the mature B cells needs to be further examined.

5. General discussion

5.1 DNA methylation

Methylation on the 5' carbon on cytosines, which are part of CpG dinucleotides is an enzymatic reaction, carried out by the enzyme family DNA methyltransferases (DNMT) (Crider, K.S. et al. 2012). The methyl donor used in the process of methylation, SAM, is a derivative of FA, which can be obtained nutritionally (Wald, D.S. et al. 2001), or by the use of probiotics (Pompei, A. et al. 2007). DNA methylation is not a transient process. The methylation profile is hereditary, ensuring the daughter cells, being the same type as the mother cell, will have the same genes available for transcription (Gowher, H. and Jeltsch, A. 2001). It was believed that methylation of a group or single CpG dinucleotides act as steric interference, inhibiting transcription factor binding to the region. Alternatively, it can act as a recruitment factor for other epigenetic mechanisms such as histone modification related proteins (Russ, B.E. et al. 2013). Changes in the methylation profile observed in the experiments conducted with the B cell line derived from a DT-40 cell line were not associated with changes in gene expression. Furthermore, while the association between FA concentration and major MHCII β chain promoter methylation was a negative one, some associations where positive. i.e., an increase in FA was associated with an increase in gene expression, as was the case with Igβ. These results strengthen the results published by others that have shown that the relationship between methylation and gene expression is not as clear cut as previously thought. Moreover, it is tissue, gene and sex specific (Anderson, O.S. et al. 2012). The lack of association between promoter methylation and gene expression was also seen in the work done with embryonic B cells, FA treatment changed the expression of Igβ while the changes in the methylation profile of the promoter region of that gene seem to be age related.

Overall, the proximal promoter methylation profile of all the genes of interest seem to be unaffected by FA treatment.

5.2 B cell receptors

5.2.1 Effect of FA on TLR2b expression and proximal promoter methylation

TLR2b, as opposed to other TLRs functions as part of a heterodimer with other TLRs (Farhat, K. et al. 2008). It recognizes a vast array of microbial associated molecular patterns (Farhat, K. et al. 2008) and may induce a strong pro inflammatory response (Cogswell, J.P. et al. 1994). Due to its potency ligands of TLR2b are used as adjuvants in several types of vaccines both in adult birds and in ovo (Barjesteh, N. et al. 2015). The proximal promoter region of chicken TLR2b has only one cytosine-guanine (CpG) dinucleotide in 200 base pairs (bp) upstream of the start codon, and the cytosine in that dinucleotide remained methylated regardless of treatment. This might indicate that this genes expression is not regulated by proximal promoter methylation. The expression of the gene, however, did increase with FA treatment in DT-40 cells, while an insignificant increase in expression was seen in cells harvested from ED21 embryos. Although it is unclear through which pathway FA treatment affected TLR2b, the observed raise in expression in B cells at day of hatch might increase the efficiency of vaccines given with TLR2b ligands as adjuvants in ovo or perhaps even immediately post hatch. This is one area in which this study hints at the immunomodulatory properties of FA.

5.2.2 Effect of FA on Igβ expression and proximal promoter methylation

The BCR is the link of the B cell to the adaptive immune system, as well as a requisite for passing the selection process that in avian species takes place in the BoF. Specifically, the signal transfer moiety made up of Igα and Igβ (Wienands, J. and Engels, N. 2001). This ability might signal the cell to activate, proliferate and secrete antibodies (Cambier, J.C. et al. 2007). In DT-40 cells an association between the proximal promoter methylation and FA concentration was observed. However, there was no significant change in expression following FA treatment. The association between FA concentration and promoter methylation was not observed in embryonic B cells. In these cells, the proximal promoter region was almost completely methylated in cells harvested from ED15 and ED18 embryos, and almost completely unmethylated in cells harvested from ED21 embryos. However, FA treatment did decrease the expression levels of Igβ in cells harvested from ED15 embryos. This might have an effect on the selection process of the B cells in the BoF, as it relies on the capability of Igα and Igβ to initiate signal transduction. This effect was not observed in the two other embryonic days examined. This result raises the possibility that the timing of treatment with FA or the developmental stage of the embryo might be factors in determining how efficient the treatment is. Research into the effect of FA on the immune system in general or specifically on the immune capabilities and development of the chicken B cell might have to take this into account.

5.2.3 Effect of FA on MHCII β chain expression and proximal promoter methylation

Endogenously, only dendritic cells, macrophages and B cells express MHCII (Boss, J.M. and Jensen, P.E. 2003, van den Elsen, P.J. et al. 2003). Presentation through this complex serves

different functions in different cells. Antigen presentation by B cells expressing this complex to CD4 positive effector T cells might lead to a humoral immune response (Hu, J. et al. 2013). In the DT-40 cell line a negative association was found between FA concentration and the percent of proximal promoter methylation of the MHCII β chain. Furthermore, the expression of the gene increased after treatment. Conversely, in embryonic B cells FA had no significant effect on MHCII β chain proximal promoter methylation or gene expression. These results are concurrent with past publications that pointed at the methylation of the class II transactivator (CIITA) gene as a mean to control MHCII expression (van den Elsen, P.J. et al. 2003). Research into the effect of FA treatment on the methylation of CIITA would help shed light on the indirect epigenetic control of MHCII expression.

5.3 The role of the BoF in chicken B cell development

The BoF is crucial for the development and maturation of the chicken B cell. Pre B cells are homing to this site (Meyer, R.K. et al. 1959), here they are selected and leave the BoF as mature naïve B cells, ready to colonize the periphery and secondary lymphoid tissues (Paramithiotis, E. and Ratcliffe, M.J. 1994). A decrease in the IgM^{low} and IgM^{med} population was observed when cells from ED15 were removed from the bursal environment. This could point to the existence of a survival signal in the BoF which the young cells are more sensitive to than the cells from the other examined ages. Conversely, the IgM^{high} population taken from the same age increased when removed from the bursal environment. This might hint at a bursal signal that keeps the B cell population in check, perhaps inhibiting proliferation. B cells have the potential to cause autoimmune diseases if they are not developed and selected properly (Grimaldi, C.M. et al. 2005).

Understanding the profile of bursal signals as embryo development progresses, as well as the sensitivity of the developing B cells to those signals should be further investigated to better understand the role of the BoF in not only B cell development, but also population control.

5.4 The effect of incubation time on embryonic B cell viability

Past publications have shown that after harvesting chicken B cells from the BoF, the cells begin to die rapidly (Compton, M.M. and Waldrip, H.M. 1998, Luna-Acosta, J.L. et al. 2015). The death rate is so high that it was impossible to carry out the experiment as was planned. By doubling the chicken serum concentration in the growth media from 5% to 10%, the cells maintained viability above 90% for longer than the time needed for the completion of the experiment. This novel growth media might present a solution for maintaining embryonic bursal B cells viable for longer periods of time, thereby allowing for more research to be done on these cells.

5.5 Conclusions

5.5.1 TLR2b, Igβ and MHCII β chain and proximal promoter methylation control

The first objective of this study was to determine if TLR2b, Igβ and MHCII β chain are under potential proximal promoter methylation control. Sequencing of the proximal promoter regions of these genes revealed that TLR2b has 1 CpG dinucleotide in this region, while Igβ has 13 CpGs and the MHCII β chain has 24 CpG dinucleotides in its proximal promoter region. Since it has been previously described that a single CpG in the promoter region has the potential to affect the expression of the gene (Maier, H. et al. 2003a), the genes examined in this study are all under potential promoter methylation control.

5.5.2 The effect of adding FA on the methylation profile of the proximal promoter region of TLR2b Igβ and MHCII β chain

As a second objective, we wanted to determine how inclusion of a methyl donor such as FA will affect the methylation profile of our genes of interest. While TLR2b promoter methylation profile was unchanged after incubation with FA regardless of cell type used, there was a discrepancy between the result obtained with the DT-40 cell line and the embryonic chicken B cells regarding Igβ and MHCII β chain. Specifically, FA addition affected the promoter methylation profile of Igβ and MHCII β chain in DT-40 cells, while this profile remained unaffected in embryonic chicken B cells. Furthermore, the promoter methylation profile of Igβ and MHCII β chain was different between the two types of cells even before FA treatment. Overall, these results are pointing at the possibility that tumoral cells have a different methylation profile and response to FA treatment than embryonic chicken B cells.

5.5.3 The effect of changes in TLR2b, Ig β and MHCII β chain proximal promoter methylation profile on gene expression

The third objective of the study was to determine whether changes in the methylation profile in the proximal promoter region belonging to TLR2b, Igβ and MHCII β chain are associated with changes in the expression of those genes. While an association between percent of promoter methylation and gene expression was not found in this study regardless of cell type, it is demonstrated in this study that addition of FA did have an effect on the expression of all three genes in the DT-40 cells, and on Igβ expression in embryonic chicken B cells. Together, the results suggest that while FA has an effect on gene expression, it is not due to changes in the proximal

promoter region of the genes in question. Whether FA addition affects the methylation of another genomic region, or through another mechanism altogether remains to be further studied.

5.5.4 Future research

The objectives described at the beginning of this thesis were fulfilled. However, there are several ways in which research into the effect of FA on chicken B cells can be improved. It is apparent from the results brought here that cell lines and normal cells respond differently to FA treatment. For that reason, results applicable for the poultry industry would be achieved using cells harvested from the organism. Furthermore, the evidence from this study suggests that during embryonic development the only factor that significantly affected the methylation profile was the age of the embryo. This is a good point in chicken embryo development to study the effect of FA as a methyl donor, as B cells are highly proliferative at this stage in the BoF, and the DNA in these cells is open to accept a methyl group. Mature naïve B cells can be induced to proliferate in vitro by adding a variety of factors to the culture. Testing the effect of FA addition on a culture of proliferating mature naïve B cells has two advantages. First, these cells can survive longer in cultures than embryonic chicken B cells can. Second, different activating factors can be used to activate the B cell. It is possible that ex vivo B cell proliferation can be achieved through signals that are exclusive to the T cell dependent or T cell independent pathways. This will help clarify the role of a methyl donor in each of the activation pathways.

It was established in this study that the changes in gene expression was not due to changes in the proximal promoter region methylation profile of TLR2b, Igβ and MHCIIβ chain. The location of these changes remains to be further studied. As it is known which of the methyl groups in FA is

used in the DNA methylation process, marking this group fluorescently or radioactively together with a full genome methylation analysis might aid in discovering where it was incorporated, and what is the role of these locations in relation to TLR2b, Igβ and MHCIIβ chain expression.

Finally, the experiments presented here are a proof of concept regarding nutritional intervention with FA in the laying hen and its effects on the progeny. It is possible that a prolonged diet supplemented with FA will have an effect on the embryo that was not detectable after only 4 hours of incubation ex-vivo. For this reason, the dietary study should be carried out, as this would mean that the embryos would be exposed to FA from the start of their development. Carrying out this study will give a better idea about the effect of FA as a nutritional factor on chicken embryonic B cell development and function in both the innate and adaptive immune systems.

5.6 Concluding remarks

Finding ways to boost the immune system of production animals such as the chicken is a beneficial endeavour for the animal itself as well as for the consumer. With the help of nutritional intervention and probiotics one can potentially raise the quality of life of these animals and lower the risk of morbidity that comes with an intensive growth used in the poultry industry. Epigenetics presents a possible double benefit, as it has the potential to aid not just the organism, but their progeny as well.

The experiments shown in this study demonstrate that FA does indeed have immunomodulatory capabilities in chicken B cells, affecting the expression of genes that are directly linked to the ability of the cell to recognize antigen. The possible explanation for the differences in results

between the cell line and the primary cell culture lies in the methylation changes occurring in cancerous cells. While the changes FA induces on immune related genes may not lie in changes to the methylation profile of the proximal promoter region, the results indicate that immune related changes in expression do occur, and that their effect on the immune capabilities of the animal, as well as their offspring should be studied further. Research into embryonic bursal B cells is made easier, as the growth media described here helps maintain these cells live for longer periods of time. This might help to learn more about chicken B cell development as well as the dynamics in the bursal environment. Past research into the role of the BoF aided in understating the role of the bone marrow in mammals (Abdou, N.I. and Abdou, N.L. 1972), the future research proposed here might aid not only in our understating of avian immunology, but general immunology as well.

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